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The Role of the Endothelin and Renin-Angiotensin Systems in Head and Neck Cancer Progression

Emma Hinsley

July 2012

A thesis submitted for the degree of Doctor of Philosophy

University of Sheffield

School of Clinical Dentistry

Unit of Oral and Maxillofacial Pathology

To grandpa

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is a frequently fatal and increasingly common epithelial malignancy. Significant morbidity and mortality frequently results from the invasion of tumours into surrounding structures and local metastasis to lymph nodes. The invasion and migration of HNSCC cells is increasingly recognised to be influenced by factors derived from adjacent tumour-associated stroma. The contextual signals regulating stromal-tumour interactions, however, remain poorly understood. Here, a role for endothelin-1 (ET-1) and angiotensin II (Ang II), in promoting pro-metastatic cross-talk between head and neck cancer cells and adjacent fibroblasts is investigated.

The results of this study indicate that treatment of normal oral fibroblasts (NOFs) with ET-1 activates ADAM17 mediated release of epidermal growth factor receptor (EGFR) ligands, triggering EGFR signalling and increased migration and invasion in neighbouring HNSCC cells. ET-1 mediated paracrine transactivation of EGFR also increases cyclooxygenase-2 (COX-2) levels in the head and neck cancer cells, providing a molecular insight into the mechanisms by which the elevated levels of ET-1 observed in HNSCC may contribute to disease progression.

Evidence is also provided that Ang II stimulates migration and invasion of HNSCC via AT_1R using a similar paracrine mechanism to that employed by ET-1, and also via an autocrine mechanism, acting on HNSCC cells alone. AT_1R is expressed by both HNSCC cells and NOFs, but not normal oral keratinocytes (NOKs). Angiotensin 1-7 (Ang 1-7), a proteolytic product of the degradation of Ang II, is able to inhibit the stimulation of HNSCC migration by Ang II and other peptides implicated in HNSCC progression including ET-1 and bradykinin (BK). Together, these results demonstrate a novel role for the RAS in head and neck carcinogenesis and implicate Ang 1-7 as a possible novel therapeutic agent in the treatment of HNSCC.

In addition, it is shown herein that ET-1 and Ang II increase the proliferation and migration of NOFs, and induce a more contractile phenotype, key features of myofibroblast transdifferentiation, a strong indicator of poor prognosis in HNSCC. Interestingly Ang II, but not ET-1, stimulates changes in gene expression indicative of myofibroblast transdifferentiation, despite both inducing similar levels of pro-invasive paracrine signalling between oral fibroblasts and HNSCC.

The findings in this thesis provide important novel information regarding the role that the ET-axis and renin angiotensin system (RAS) play in contributing to HNSCC progression and the results collected identify potential novel targets and agents for drug therapies in the treatment of the disease.

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List of Figures and Tables

Abbreviations

ACE	angiotensin converting enzyme
ACE2	angiotensin converting enzyme 2
ADAMs	a disintegrin and metalloproteinase protein
AREG	amphiregulin
AT_1R	angiotensin receptor 1
AT_2R	angiotensin receptor 2
Ang 1-7	angiotensin 1-7
Ang I	angiotensin I
Ang II	angiotensin II
B1R	B1 receptor
B2R	B2 receptor
BAD	BCL-2-associated death promoter
BCL	B-cell lymphoma
BGF	basic growth factor
BFGF	basic fibroblast growth factor
big ET	big endothelin
BK	bradykinin
BSA	bovine serum albumin
CAFs	cancer associated fibroblasts
cAMP	cyclic adenosine monophosphate
CDK	cyclin dependant kinase
cDNA	complementary DNA
COX-2	cyclooxygenase 2
CTGF	connective tissue growth factor
CXCR4	cxc chemokine receptor 4
DMEM	dulbecco's modified eagle's medium
DUSP1	dual specificity protein phosphatase 1
ECE	endothelin-converting enzyme
ECM	extracellular matrix

EDTA	ethylene-diaminetetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGFRvIII	epidermal growth factor receptor variant III
EMT	epithelial to mesenchymal transition
EPCs	endothelial progenitor cells
ERK	extracellular signal regulated kinase
ET	endothelin
ET_AR	endothelin receptor A
ET_BR	endothelin receptor B
FBS	foetal bovine serum
FcγR	Fcγ receptor
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
g	relative centrifugal force
GAG	glycoaminoglycan
GPCR	G protein coupled receptor
GRP	gastrin-releasing peptide
h	hour
HB-EGF	heparin bound-epidermal growth factor
HEK	human embryonic kidney
HGF	hepatocyte growth factor
HIF-1	hypoxia inducible factor-1
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
HSV	herpes simple virus
IGF-1	insulin like growth factor-1
IGF-1R	insulin like growth factor-1 receptor
IL	interleukin
JAK	janus kinase
JNK	jun N-terminal kinase

kDa	kilodalton
LHRH	luteinizing hormone-releasing hormone
LOH	loss of heterozygosity
LPA	lysophosphatidic acid
M	molar
MAPK	mitogen-activated protein kinase
MasR	mas receptor
MCP1	monocyte chemotactic protein 1
MEK	mitogen activated extracellular signal regulated kinase
min	minute
miRNA	microRNA
ml	millilitre
mM	millimolar
MMPs	matrix metalloproteases
Mrg	mas-related gene
mRNA	messenger RNA
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NEP	neprilysin
NF-κB	nuclear factor-kappa B
NHEK	normal human embryonic keratinocytes
NK1R	neurokinin-1 receptor
ng	nanogram
nM	nanomolar
NOF	normal oral fibroblast
NOK	normal oral keratinocyte
NTSR1	neurotensin receptor 1
OSCC	oral squamous cell carcinoma
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PDGF	platelet-derived growth factor
PGE_2	prostaglandin E2
PI3-K	phosphatidylinositol 3-kinase
PLC	phospholipase C
PTEN	phosphatase and tensin homolog deleted on chromosome 10
qPCR	real time polymerase chain reaction
RAS	renin-angiotensin system
Rb	retinoblastoma gene
rho/ROCK	rho-associated protein kinase
RIPA	radioimmunoprecipitation assay
RISC	RNA induced silencing complex
ROS	reactive oxygen species
RPMI-1460	roswell park memorial institute medium
s	seconds
SDF-1	stromal cell-derived factor 1
SMC	smooth muscle cells
SNPs	single nucleotide polymorphisms
S.O.C	super optimal broth with catabolite repression
STAT	signal transducer and activator of transcription
TACE	tumor necrosis factor-α-converting enzyme
TAE	tris acetate EDTA
TGF-α	transforming growth factor-alpha
TGF-β	tumour growth factor-beta
TGF-βR	tumour growth factor-beta receptor
TIMPs	tissue inhibitors of metalloproteinases
TJ	tight junctions
TLR	toll-like receptor
TNF-α	transforming neurosis factor-alpha
TNM	tumour/nodes/metastasis
UK	United Kingdom
uPA	urokinase plasminogen activator

UTR	untranslated region
UV	ultraviolet
V	volts
VEGF	vascular epidermal growth factor
v/v	volume/volume
w/v	weight/volume
ZO-1	zonula occludens
α-SMA	alpha-smooth muscle actin
μg	microgram
μl	microlitre
μm	micrometre
μM	micromolar

Publications

Hunt, S., Jones, A. V., Hinsley, E. E., Whawell, S. A., & Lambert, D. W. (2011) MicroRNA-124 suppresses oral squamous cell carcinoma motility by targeting ITGB1, *Febs Letters*, **585**:187-192

Hinsley, E. E., Hunt, S., Hunter, K. D., Whawell, S. A., & Lambert, D. W. (2012) Endothelin-1 stimulates motility of head and neck squamous carcinoma cells by promoting stromal-epithelial interactions. *International Journal of Cancer*, **130**:40-47

Hinsley, E. E., Kumar, S., Hunter, K. D., Whawell, S. A., & Lambert, D. W. (2012) Endothelin-1 stimulates oral fibroblasts to promote oral cancer invasion. *Life Sciences,* Article in press

Chapter 1: Introduction

1.1 Cancer

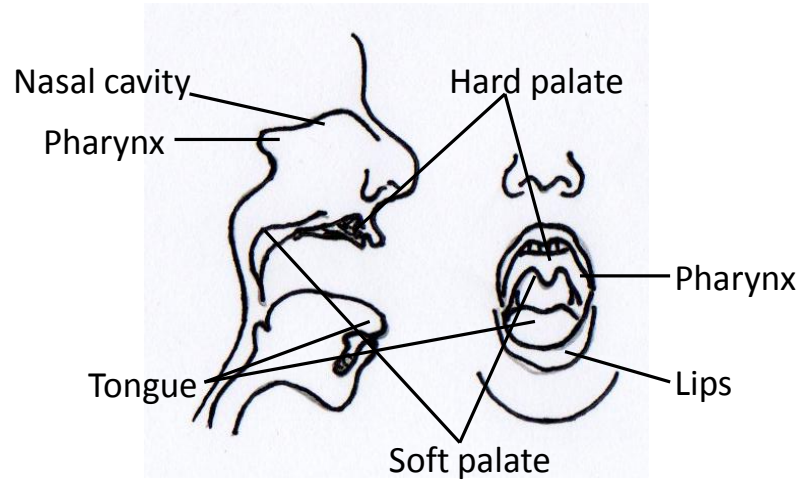
A cancer is a complex of tissues that contains multiple and distinct cell types that interact with one another. Normal cells are recruited to the tumour microenvironment and form the reactive stroma. These recruited cells are nearly always active participants in cancer progression and play important roles in tumourigenesis (Hanahan and Weinberg, 2011). Cancer is a multi-component process and six biological capabilities have been described as being paramount within this process (Hanahan and Weinberg, 2011). Both the tumour cells and those found within the surrounding tumour microenvironment can contribute to the acquirement of some of these hallmark characteristics (Hanahan and Weinberg, 2011). The six hallmarks of cancer include sustaining proliferative signalling, resisting cell death, inducing angiogenesis, evading tumour suppressors, activating invasion and metastasis and enabling replicative immortality (Hanahan and Weinberg, 2000). Genomic instability is an important factor that allows some of these characteristics to be achieved (Hanahan and Weinberg, 2011).

1.2 Head and neck cancer

Head and neck cancers originate within the tissues and organs located within the head and neck. The term head and neck cancer is used to describe a number of different cancer types including eye cancer, nasal and paranasal sinus cancer, nasopharyngeal cancer, mouth and oropharyngeal cancer, laryngeal cancer and oesophageal cancer. This project focuses on mouth and oropharyngeal cancer which describes any cancers found located within the oral cavity including those identified on the front two-thirds of the tongue, gums, cheeks, lip, floor and roof, described as the palate, of the mouth and behind the wisdom teeth. The pharynx is the clinical name used to describe the throat. The throat is divided into three areas and the area found directly at the back of the mouth is called the oropharynx (Figure 1.1A). Cancers that originate within this area are described as oropharyngeal cancers (Cancer Research UK, 2012). The term head and neck cancer will be used in this thesis to describe mouth and oropharyngeal cancer.

Head and neck cancer is a complex disease made even more difficult to treat because of its prominent and distinguishable location associated with the anatomy of the patient. The main treatment methods for head and neck cancer have remained unchanged for years and there has been little improvement in survival rates over the past three decades, which have remained stubbornly low at 50% (Cancer Research UK, 2012). Treatments include surgery which can result in severe disfigurement and emotional trauma for the patient and courses of radiotherapy and chemotherapy (Scully and Porter, 2000). Early diagnosis is the major factor in achieving a good prognosis from head and neck cancer (Shah and Gill, 2009;

A



B **C**

Figure 1.1 Mouth and oropharyngeal cancer: A schematic diagram highlighting the organs and tissues in which head and neck cancers can originate. The term head and neck cancer is used to describe a number of different cancer types including eye cancer, nasal and paranasal sinus cancer, nasopharyngeal cancer, mouth and oropharyngeal cancer, laryngeal cancer and oesophageal cancer **(A)**. Photographs of a head and neck carcinoma located on the floor of the mouth **(B)** and a local node metastasis **(C)** (Photographs courtesy of Dr. K. Hunter).

Nagpal and Das, 2003). Many patients suffering from head and neck cancer show no symptoms in the early stages of the disease therefore making it much more difficult to diagnose it at an early stage (Cancer Research UK, 2012). Symptoms can include a sore or ulcer within the mouth, a lump or thickening of the mouth, throat or tongue, or the observation of patches of red or red and white within the patient's mouth that do not disappear (Scully and Porter, 2000a). The patient can also experience difficulty when chewing or swallowing and they may begin to experience persistent pain. The symptoms experienced by the patient can vary depending on the position and size of the malignancy (Scully and Porter, 2000b).

The TNM system is used as a clinical staging system in the diagnosis and prognosis of head and neck cancer. It was first devised by Pierre Denoix between 1943 and 1952 in order to stage and grade all solid tumours. The system is made up of three parameters; T represents the size of the lesion and whether it has invaded into nearby tissue, N describes the regional lymph nodes that are involved and it represents the extent and the distribution of metastases within the regional lymph nodes and M represents the presence or absence of further distant metastases (Figure 1.2A) (La Vecchia *et al*, 1997). Clinical staging of HNSCC is determined by the TNM system. The prognosis of a patient decreases as the clinical staging of the tumour increases (Figure 1.2B).

The majority of mouth and oropharyngeal cancers are squamous cell carcinomas. The oral mucosa is the term used to describe the tissue that lines the inside of the mouth, nose, throat and larynx. The oral mucosa is divided into three main layers which include the epithelium, the basement membrane and connective tissue (Figure 1.3). The epithelium consists of tightly packed epithelial cells which form a dense, wall like structure. The epithelium creates a somewhat permeable barrier and aids to protect the tissue underneath. The oral epithelium is defined as a stratified, squamous epithelium which is further divided into three more layers. The top lay of the epithelium is described as the superficial layer which consists of a single layer of cuboidal epithelial cells. Spinous or prickle cell layers are found beneath the superficial layer and the final layer of the epithelium is called the basal layer. Melanocytes and merkel cells can be found within this layer. These layers can also display a variability of keratinisation which is the term used to describe the deposition of keratohyalin granules within the cells resulting in the formation of an impermeable layer (Moss-Salentijn and Hendricks-Klyvert, 1990). The basement membrane is located beneath the epithelium and provides support for the epithelial cells. It also helps to connect the epithelium to the connective tissue. The connective tissue found beneath the basement membrane also helps to provide structural support. The connective tissue is composed of various cell types including

A

The TNM System

Tumour:

T1	*Greatest diameter of primary tumour is 2 cm or less*
T2	*Greatest diameter of primary tumour is at least 2 cm but does not reach 4 cm*
T3	*Greatest diameter of primary tumour is 4 cm*
T4	*Massive tumour which is at least 4 cm in size with gross local invasion*

Nodes:

N0	*No clinically positive nodes are present*
N1	*A single ipsilateral node is present but is not greater than 3 cm in diameter*
N2	*a) A single ipsilateral node is present and is at least 2 cm in diameter but does not reach 6 cm* *b) Multi ipsilateral nodes are present but do not reach 6 cm in diameter* *c) Bilateral or contralateral nodes are presentbut do not reach 6 cm in diameter*
N3	*Any node which is at least 6 cm in diameter*

Metastasis:

M0	*No distant metastases are present*
M1	*Distant metastases are present*

B

Clinical Staging

Stage 1:		
T1	N0	M0
Stage 2:		
T2	N0	M0
Stage 3:		
T3	N0	M0
T1, T2 or T3	N1	M0
Stage 4:		
T4	N0 or N1	M0
any T	N2 or N3	M0
any T	any N	M1

Figure 1.2 The TNM system and clinical staging associated with head and neck cancer: The TMN system is made up of three parameters. T represents the size of the lesion, N represents the extent and the distribution of the metastases within the regional lymph nodes and M represents the presence or absence of further metastases **(A)**. Clinical staging of HNSCC is determined by the TNM system **(B)**. The prognosis of a patient decreases as the clinical staging of the tumour increases (Figure adapted from La Vecchia *et al*, 1997).

immune cells, fibroblasts and endothelial cells which form blood vessels. These cellular components are all held together by the extra cellular matrix (ECM).

Head and neck squamous cell carcinoma (HNSCC) can arise from clinically normal oral mucosa but normally the disease is often preceded by a precancerous or potentially malignant lesion. Leukoplakia is the most common form of precancerous lesion but eryhroplakia also exists (Scully and Porter, 2000a). Leukoplakia is defined by the World Health Organisation as 'a white patch on the oral mucosa which cannot be rubbed off and cannot be ascribed clinically or histologically, to any other diagnosis'. Leukoplakia typically occurs on the buccal mucosa, lateral border of the tongue or on the floor of the mouth. Areas of the lesion can be well defined or can diffuse and cover large areas of the mucosa. The colour of the lesion can vary from 'pearly' white to yellowish or greyish-white. The outline of the lesion can also vary as can the texture. The lesion can be slightly raised, flat or smooth or it can be a thick, verrous, fissured lesion that is firm on palpation. Leukoplakia can be classified as homogenous or non-homogenous. The majority of leukoplakias are benign and are a result of the thickening of keratin layers. The remaining examples are described as epithelial dysplasias and are characterised by atypical cytological features. The term dysplasia is used to describe areas of the epithelium which exhibit cellular and architectural changes. These can include an abnormal change in cell size and shape, nuclear size and shape, an increased nuclear/cytoplasmic ratio, increased or abnormal mitotic figures within the nucleus or an increase in the size and number of nucleoli. Architectural changes include loss of polarity, increased cell density, basal cell hyperplasia and a disordered maturation from basal cells to squamous cells. Dysplasias do not invade the basement membrane. They can be described as mild through to severe which represents the depth of epithelium into which it effects. Mild dysplasia effects the lower third of the epithelium, moderate the middle and sever describes the involvement of the upper third. When the dysplasia becomes so severe that it affects all of the epithelium, from the bottom through to the top, the lesion is described as a carcinoma *in situ*. Only around 5% of leukoplakias become malignant (Rodrigues *et al*, 1998). The presence and severity of dysplasia within the oral mucosa of a patient cannot be used as a reliable guide for the treatment of individual cases; however its possible link to the development of malignancy makes it a necessary protocol (Fleskens and Slootweg, 2009). At present there are no consistent markers or indicators to determine malignant progression. Erythroplakia is another pre cancerous legion and is defined by the World Health Organisation as 'a red patch on the oral mucosa which cannot be rubbed off and cannot be ascribed clinically or histologically, to any other diagnosis'. Erythroplakias are extremely rare and are nearly always dysplastic (Porter and Scully, 2000a; Scheifele and Reichart, 2003; Rodrigues *et al*, 1998).

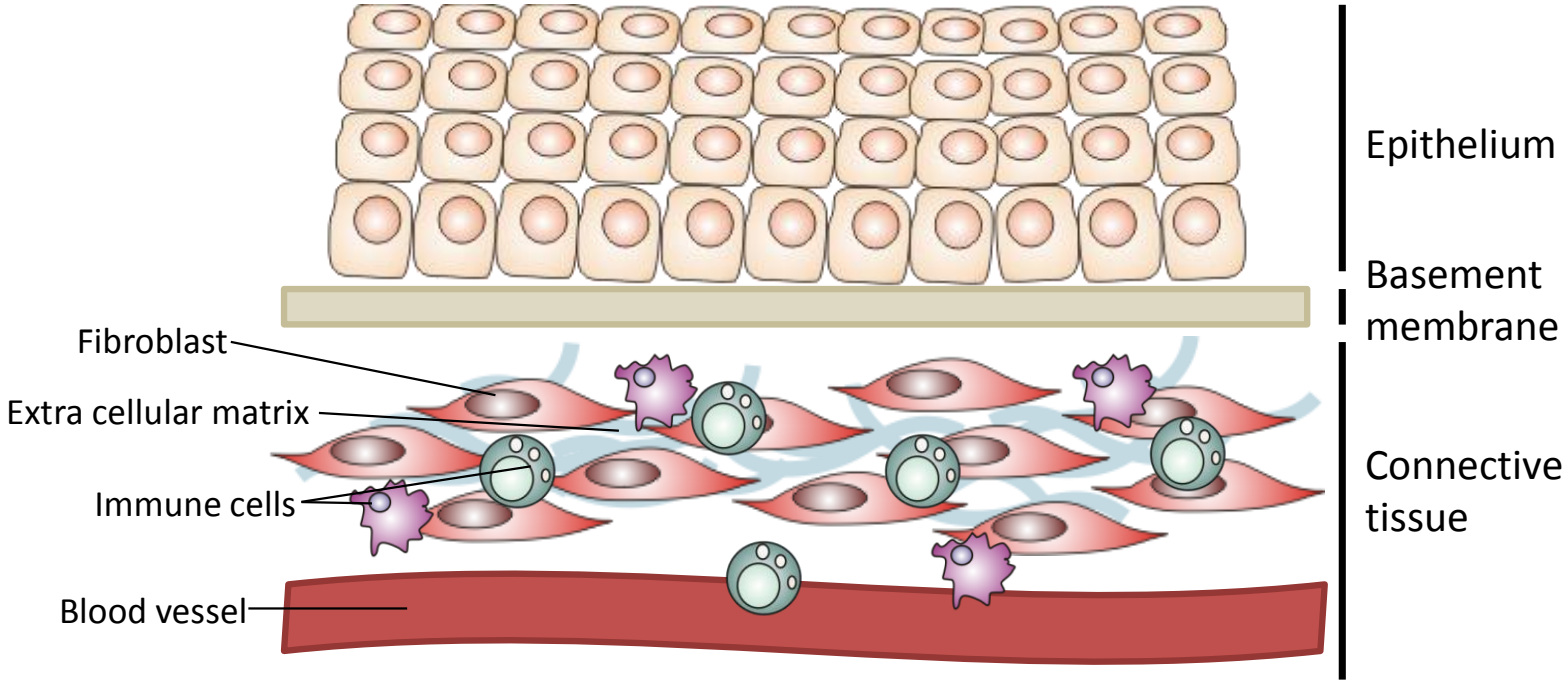


Figure 1.3 Structure of the oral mucosa: A schematic diagram of the oral mucosa indicating the major structures including the epithelium, basement membrane and connective tissue. The epithelium is made up of stratified squamous epithelial cells that form a well defined, organised, wall like structure. The phenotype of the epithelium can vary depending on its location within the oral cavity. The epithelium can be differentiated or non-differentiated, keratinised or non-keratinised. The basement membrane provides support for the epithelium and connects to the connective tissue found beneath. The connective tissue is made up of a number of different cell types including fibroblasts, endothelial cells and various immune cells. The ECM holds the components of the connective tissue together.

1.2.1 Metastasis in head and neck cancer

In patients presenting with HNSCC, local metastasis to the lymph node and invasion into surrounding bone are both common factors associated with the disease. Metastatic spread is also common in patients who present with locally advanced stage HNSCC and is often common when HNSCC is diagnosed at a late stage. There are very few treatment options for patients presenting with metastatic disease. Only a third of patients who present with metastatic disease will respond to palliative chemotherapy. The majority will not and their survival may be as short as five to nine months (Bhave *et al*, 2011).

The metastatic spread of any cancer is a complex and multi component process. The tumour cells responsible for metastatic spread from the primary tumour must possess the ability to intravasate from the primary tumour into the circulatory system, be able to survive within it and have the capability to extravasate at a distant site and be able to proliferate within this foreign environment. The cell must undergo and escape a number of cellular processes in order to be able to metastasise successfully. These include the ability to be able to increase their migratory and invasion potential and to be able to avoid apoptosis (Bhave *et al*, 2011).

The invasion and metastasis of cancer cells is achieved by a number of different mechanisms which include the presence and action of certain tumour-associated proteases that have the ability to destroy the surrounding matrix and basement membranes of the cancerous cells (Noel *et al*, 1997; Johnson *et al*, 1998). An example of this is the matrix metalloproteinase (MMP) family which contains 18 members. The members that have been associated with ovarian cancer include MMPs 2, 3, 7, 9 and 13 and all of these when present have correlated with the ovarian carcinoma cells having high invasive and metastatic potential (Rosanò *et al*, 2001).

The conversion of epithelial cells to mesenchymal cells is an important transition and also plays a vital role in tumour progression by promoting cellular migration and invasion. Epithelial to mesenchymal transition (EMT) can include a number of characteristic changes to occur within a cancer cell resulting in the cell obtaining a fibroblastic invasive phenotype. With this transition the cell begins to down regulate epithelial-specific proteins including tight junction (TJ) proteins and adherens. This is accompanied with a reduction in the expression of E-cadherin (Cano *et al*, 2000; Batlle *et al*, 2000). The intracellular domain of E-cadherin can bind to and interact with a number of catenin proteins including α-, β-, γ- and p-120 catenin. The catenins can connect the adhesion complex to the actin cytoskeleton (Thiery, 2002). To create a functional and complete adherens junction the catenins that are joined to the actin

cytoskeleton must bind to the cytoplasmic tail of cadherins. If the junction is not completed correctly or is disrupted then β-catenin may be released from the adherens junction pool. β-catenin, if not degraded correctly, has the ability to translocate to the nucleus and trigger the transcription of a variety of genes that can contribute to cancer progression including c-myc and cyclin D1. The loss of E-cadherin therefore results in the increased abundance of β-catenin; it may be this increase that contributes to the induction of genes that contribute to and promote cell migration, invasion, proliferation and EMT (Savagner, 2001). Zonula occludens-1 (ZO-1) is a 220 kDa scaffold protein that can interact with specific sites on the plasma membrane and also with other proteins (Tsukita *et al*, 2001). It has been suggested that ZO-1 may play a part in connecting E-cadherin to the actin cytoskeleton (Itoh *et al*, 2001). In has been experimentally shown in nude mice that the phenotypic alterations that arise are associated with EMT and increased tumour progression and that this in turn may be caused by the cytosolic localization of ZO-1.

In contrast to the loss of specific markers which are associated with an epithelial phenotype the cell can begin to produce mesenchymal markers including vimentin and N-cadherin. The increase in N-cadherin is deemed to be responsible for the increase in invasion and metastatic ability of the cancerous cell therefore highlighting its possibility as a therapeutic target in the treatment of cancer (Cavallaro and Christofori, 2004; Huber *et al*, 2005).

1.2.2 Incidence and epidemiology

In 2009, statistics showed that in the United Kingdom (UK) 6,236 people were diagnosed with head and neck cancer (Cancer Research UK, 2012) and during 2010 the cancer accounted for around 1,985 deaths (Cancer Research UK, 2012). Head and neck cancer contributes to 3% of all cancers diagnosed in the UK (Cancer Research UK, 2012) and is the 12th most common cancer in men and the 16th most common cancer in women (Cancer Research UK, 2012).

Each year approximately 600,000 people are diagnosed worldwide with head and neck cancer (Parkin *et al*, 2005). In some parts of the world HNSCC can contribute up to 25% of all cancers diagnosed. The common incidence of HNSCC in these countries is often due to the indulgence of the population in high risk factors including tobacco smoking and betal quid chewing. Two thirds of all head and neck cancer cases are diagnosed in people living in developing countries. These countries include Sri Lanka, which has the greatest number of males presenting with head and neck cancer per 100,000 people in the population, Botswana, Bangladesh and India (Cancer Research UK, 2012). Within the European Union 66,650 new cases of the disease are

diagnosed each year (Cancer Research UK, 2012), with the most cases occurring in France and closely followed by Spain.

Head and neck cancer is more common in males compared to females; however this sex incidence ratio has decreased in the past 50 years from 5:1 to 2:1 (Cancer Research UK, 2010). In the UK the risk of developing oral cancer increases with age. 86% of all oral cancer cases occur within people over the age of 50 (Cancer Research UK, 2010). In developing countries head and neck cancer can be common within younger generations too and many European countries and in the US there has been an increase in the number of young and middle-aged men being diagnosed with cancer of the tongue (Annertz *et al*, 2002; Schantz and Yu, 2002; Llewellyn *et al*, 2001).

Since 1975 there has been a reduction in the number of men above the age of 80 being diagnosed with head and neck cancer (Cancer Research UK, 2010). The number has decreased from 70 per 100,000 people in 1975 to around 35 per 100,000 people in 2006. This observation however has not been observed in men over the age of 70 and within this age group the incident rate has remained stable (Cancer Research UK, 2010). There has been an alarmingly large increase in the number of head and neck cancer diagnoses observed in men who fall between the ages of 40-50 (Cancer Research UK, 2010). This statistic highlights that HNSCC is an arising problem in younger people and therefore there is a pressing need to understand the molecular mechanism involved in its development and progression in order to define new therapeutic targets that could be used in the treatment of the disease.

1.2.3 Risk factors

There are several predisposing risk factors associated with head and neck cancer: tobacco usage, alcohol consumption, diet, oral health, exposure to ultraviolet light and exposure to viruses.

1.2.3.1 Tobacco usage

Tobacco usage is a major risk factor of head and neck cancer. The main carcinogenic agents present and active in all methods of tobacco consumption are thought to be nitrosamines which are derived from nicotine (Johnson, 2001). The production of tobacco smoke from all consumption methods including cigarette, pipe and cigar smoking, has been linked to HNSCC. The risk of developing the disease increases with the number of cigarettes and cigars smoked per day along with the amount of tobacco smoked through a pipe and the length of time a person smokes for during their lifetime (Johnson, 2001).

Reverse smoking, a habit associated with many communities in India, is the practice in which the lit end of a cigarette is placed within a person's mouth. This type of inhalation is associated with the risk of developing cancer of the palate which is one of the rarest forms of head and neck cancer (Gupta *et al*, 1984).

Snuff is a type of tobacco that can be inhaled and placed within the oral mucosa in the buccal and labial sulci. The practice is known as snuff dipping. The habit is common within the south eastern states of the US and Sweden and is thought to be one of the reasons why head and neck cancer cases have increased within young people (Johnson, 2001).

The habit of paan chewing is practiced by 200 million people around the world and is most common in communities within South-East Asia and India, and in ethnic groups from these regions who now live in the UK (Johnson, 2001). The paan, also described as betal quid, is made up of a betal leaf which is wrapped around a betel nut and slaked lime with the addition of tobacco and various spices. The practice of paan chewing involves placing the quid within the buccal sulcus and leaving it there for long periods of time. The area in which the quid is held within the mouth often develops leukoplakia.

1.2.3.2 Alcohol consumption

The role that alcohol plays in the development of head and neck cancer is controversial (Ogden and Wight, 1998). It has been suggested that the presence of carcinogenic contaminants and congeners in alcoholic drinks may be responsible for the enhanced ability of the carcinogens to cross the oral mucosal barrier within the mouth (Ogden and Wight, 2008). The barrier's function may also be impaired by the lifestyle habits associated with heavy drinkers including a poor diet and an impaired metabolism. The consumption of large quantities of alcohol is also thought to affect the liver's ability to detoxify possible carcinogens correctly and to suppress the natural immune response of the body. Both factors can contribute to the risk of developing cancer. Mouthwash usage has also been linked to head and neck cancer and those that contain a high alcohol content of 25% or over are thought to be the most dangerous (Ogden and Wight, 2008).

1.2.3.3 Diet and nutrition

Iron is a vital element needed for the production and maintenance of a correctly functioning oral epithelium. Iron-deficiency anaemia has been recognized as a risk factor for the development of head and neck cancer and is thought to be responsible for making the oral mucosa more susceptible to damage from carcinogens (Winn, 1995). Vitamin A is a fatty acid that is required for the maintenance of the stratified squamous epithelium of the oral mucosa, and a diet high in antioxidant vitamins A, C and E is thought to lead to a reduced risk of

developing head and neck cancer (Winn, 1995). This risk in greatly reduced by consuming a diet high in fresh fruit and vegetables.

1.2.3.4 Oral health

It has been difficult to fully understand the extent of the risk that poor oral hygiene has on the development of head and neck cancer. Many patients suffering with the disease have poor dentitions but they are also heavy smokers and drinkers and therefore it is difficult to link the extent of the damage caused by the poor state of the mouth alone (Shah and Gil, 2009). People with poor oral health also visit the dentist less regularly and therefore have a slightly increased risk of developing head and neck cancer (Cancer Research UK, 2012).

1.2.3.5 Ultraviolet light

Squamous cell carcinoma of the lip is the only cancer of the mouth in which ultraviolet (UV) light plays a part in its development. It shows a different geographical distribution from other head and neck cancers and is most commonly found within the white population living in Australia and Canada (Gorsky and Epstein, 1998; Hicks and Flaitz, 2000). These countries experience high levels of sunshine throughout the year and lip cancer alone can contribute up to 50% of all head and neck cancer diagnosed (Gorsky and Epstein, 1998; Hicks and Flaitz, 2000). The cancer is much more likely to occur on the lower lip and is more common in men compared to women (Gorsky and Epstein, 1998; Hicks and Flaitz, 2000). Higher levels of the melanin pigment in the skin of dark-skinned people act as a barrier against the damage caused by UV light and therefore the number of cases of lip cancer that occurs within this group of people is relatively rare.

1.2.3.6 Viruses

1.2.3.6.1 Human papillomavirus

The human papillomavirus virus (HPV) has been recognised as a risk factor in the aetiology of cancer of the cervix, another form of squamous cell carcinoma (Woods *et al*, 1993; Miller and White, 1996). The two types of virus associated with the disease, type 16 and 18, have been shown to increase in frequency in the transition of the oral mucosa from its normal form to a dysplastic form and on to a carcinoma. It has been suggested that the virus affects the normal mucosa, by triggering its differentiation to a dysplastic phenotype. The proteins that the virus codes for are thought to be able to bind to the tumour-suppressor genes, p53 and retinoblastoma (Rb) gene causing their inactivation or mutation and ultimately affecting their natural role within the mucosa (Woods *et al*, 1993; Miller and White, 1996). HPV is an independent risk factor for oropharyngeal cancer (Ang *et al*, 2010). Patients with HPV-positive HNSCC have an improved prognosis in comparison to patients with HPV-negative HNSCC (Ang

et al, 2010; Weinberger *et al*, 2006), in part due to their ability to respond better to radiology and chemotherapy (Fakhry *et al*, 2008).

1.2.3.6.2 Herpes simplex virus

It has been observed that the herpes simple virus (HSV) can be carcinogenic and cocarcinogenic (Scully, 1993) and elevated levels of the virus have been associated with HNSCC (Larssen *et al*, 1991; Shillitoe *et al*, 1982; Kumari *et al*, 1982). This evidence therefore suggests that the virus could play a role in the aetiology of head and neck cancer.

1.2.3.7 Genetic alterations

There are a number of genetic alterations that have been identified in the development and progression of HNSCC. These alterations can vary among patient cases and ultimately lead to the uncontrolled and irregular proliferation of epithelial cells and clonal expansion.

Genetic alterations can include the loss of chromosomal material from specific areas on specific chromosomes, a process known as loss of heterozygosity (LOH) (Field, 1992; Scully, 1993). Different LOH are associated with different stages of histological progression as the oral epithelium changes from its normal, stratified, well defined and organised formation through to a dysplastic form and finally a carcinoma which can include local and distant metastatic properties and invasion into the surrounding bone. The LOH associated with a predysplastic lesion occurs at 9p (Field, 1992; Scully, 1993). A LOH at 3p or 17p can cause a transition from the predysplastic lesion to a dysplastic lesion (Field, 1992; Scully, 1993). An additional LOH at 11q, 13q or 14q can lead to the formation of a carcinoma and the addition of LOH at 6p, 8 or 4q can cause invasion (Field, 1992; Scully, 1993). The areas of LOH identified have also been identified as areas of chromosomes containing known and presumed tumour suppressor genes including the p53 gene at 17q and the Rb gene at 13q (Field, 1992; Scully, 1993).

Tumour-suppressor genes are important in controlling the growth of a cell. The genes encode for growth inhibitory proteins and in normal circumstances they work in balance with oncogenes, which encode growth-promoting genes, to ensure controlled cell proliferation and growth. During carcinogenesis however, a tumour-suppressor gene could be mutated or its products could be inactivated. A proto-oncogene could under the same circumstances also be mutated causing the activation of an oncogene or the enhanced activity of an already activated oncogene. In both circumstances the control of correct cellular proliferation could be lost and enhanced tumour growth and tumour formation could be triggered.

The p53 tumour suppressor gene is located on chromosome 17q13 and is frequently mutated in many cancer cases including head and neck cancer and has been detected in over 50% of all cases diagnosed (Field *et al*, 1993; Brachman *et al*, 1992; Hoffmann *et al*, 2008). The normal function of the p53 protein, the product of the p53 gene, is to detect DNA damage within the cell and to arrest the cell cycle and allow correct repair or destruction, via apoptosis, of the damaged DNA. Inactivation of the gene results in cells being unable to respond to DNA damage and stress, resulting in uncontrolled cellular growth due to a loss of cell cycle inhibition and a lack of apoptosis (Vogelstein *et al*, 2000).

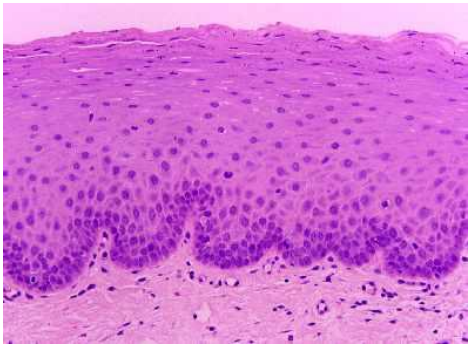
The c-myc and ras families are both examples of oncogenes which have been implicated in the progression of head and neck cancer. The families are responsible for the production of many growth-promoting proteins which include stimulatory cell-cycle regulating proteins, growth factor receptors and signal-transmitters. Many of these are involved in the activation and maintenance of the cell cycle particularly through the G_1 phase and into the S phase, the phase in which DNA synthesis takes place. During carcinogenesis the over production or mutation of these oncogene products can lead to uncontrolled cell growth and proliferation (Scully, 1992).

It is common to observe a truncated form of the epidermal growth factor receptor (EGFR); EGFR variant III (EGFRvIII). This truncated form of the receptor has an in-frame deletion of exons two to seven which results in a shorter extracellular domain (Sugawa *et al*, 1990; Bigner *et al*, 1990). This mutation results in the receptor being constitutively active in a ligand-independent manner. This mutation occurs in approximately 40% of all HNSCC cases (Sok *et al*, 2006) and is known to result in increased cell proliferation, migration invasion and *in vivo* tumour growth (Wheeler *et al*, 2006). The mutation is associated with increased levels of signal transducer and activator of transcription (STAT) 3 (Wheeler *et al*, 2010) and is known to be able to transform and enhance the motility of normal mouse fibroblasts *in vivo* (Pedersen *et al*, 2004). STAT3 is a transcription factor and is found downstream of EGFR and EGFRvIII. It plays a role in mediating EGFR signalling and can regulate MMP-2 and MMP-9 gene expression. Constitutive activation of STAT3 is associated with HNSCC (Leeman *et al*, 2006) and results in activation of genes including B-cell lymphoma (BCL)-XL, vascular endothelial growth factor receptor (VEGF) and cyclin D1 which promote cell processes including apoptosis, angiogenesis and cell cycle progression (Klein and Grandis, 2010).

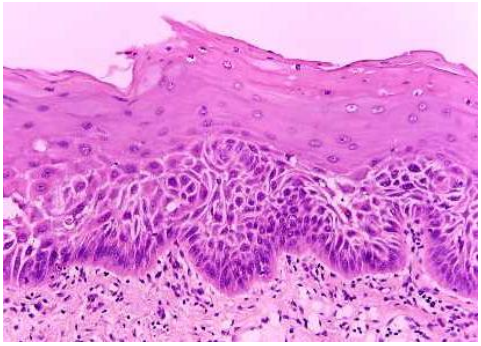
1.2.4 Classification and pathology

HNSCC pathiogenesis is a multistep process which is a result of the accumulation of genetic

A: Oral mucosa



B: Dysplasia



C: Carcinoma

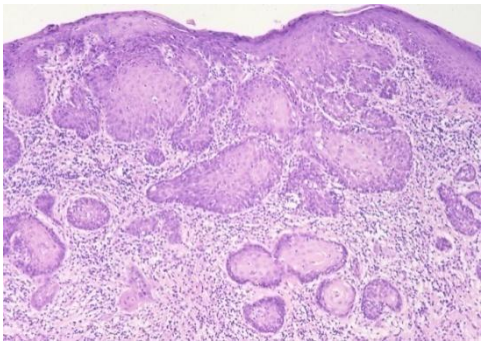


Figure 1.4 Haematoxylin and eosin stained sections of taken from a normal oral mucosa, dysplastic mucosa and a carcinoma of the oral mucosa: The sections highlight the changes in the structure of the oral mucosa associated with the progression of HNSCC. The loss of organisation is clear from that of the normal mucosa, original magnification x200 **(A)**, through to a dysplastic mucosa, original magnification x200 **(B)** and nests of squamous epithelium invading the underlying tissues, original magnification x40 **(C)**. A number of histological features are recorded which may impact on the patient's treatment options and prognosis (Photographs courtesy of Dr. K. Hunter).

mutations and histological changes within the oral mucosa. These changes result in the alteration of the oral mucosa resulting in the gradual disappearance of its organised, well defined, stratified epithelial layer and the accumulation of the disorganisation and loss of cellular structures associated with dysplasia and carcinoma (Figure 1.4).

There are three general clinical classifications for head and neck cancer. Stage I/II is described as early-stage disease. This classification of cancer is often treated with surgery and/or radiation. Stages III/IV is described as locally advanced disease and two-thirds of all patients that present with HNSCC will present at this stage (Grandis *et al*, 2004). In order to treat this stage of the disease it is often common practice to combine surgery with radiation and/or chemotherapy. The final classification is recurrent/metastatic disease and palliative chemotherapy is the most common form of treatment offered to patients.

HNSCCs can be graded according to their degree of differentiation. The cancer can be well differentiated, where the neoplastic epithelium is clearly of squamous type and masses of prickle cells are present and there is still a limiting layer of basal cells which are situated around the periphery (Nagpal and Das, 2003). A tumour can be described as being moderately differentiated. In this example the cells within the tumour show less keratinisation compared to the extent seen in the well differentiated tumour and there is more mitotic activity and nuclear and cellular pleomorphism. The cells however are still identified as squamous in type (Nagpal and Das, 2003). A poorly differentiated tumour shows hardly any keratinisation and in some examples it is even absent completely. There is prominent nuclear and cellular pleomorphism. Immunohistochemistry can be used to detect cytokeratins if the epithelial cells are so distorted that they have become unrecognizable (Nagpal and Das, 2003). The importance of the non-epithelial components of the area which immediately surrounds the tumour cells is paramount when determining the differentiation stage of a HNSCC. This area is described as the tumour microenvironment.

1.3 Tumour microenvironment

Tumour cells do not exist alone. The development and progression of epithelial tumours is profoundly influenced by the surrounding tumour microenvironment (the reactive stroma). The tumour microenvironment is an intricate system of many cell types including endothelial cells, smooth-muscle cells, macrophages, dendritic cells and fibroblasts. The tumour microenvironment does not only play a benign, supportive role in cancer progression but studies have identified that a reactive, transformed stroma can initiate and promote malignant changes in the epithelial cells found adjacent to it (Stover *et al*, 2006). In addition to this phenomenon malignant epithelial cells can stimulate non-transformed stroma resulting in its

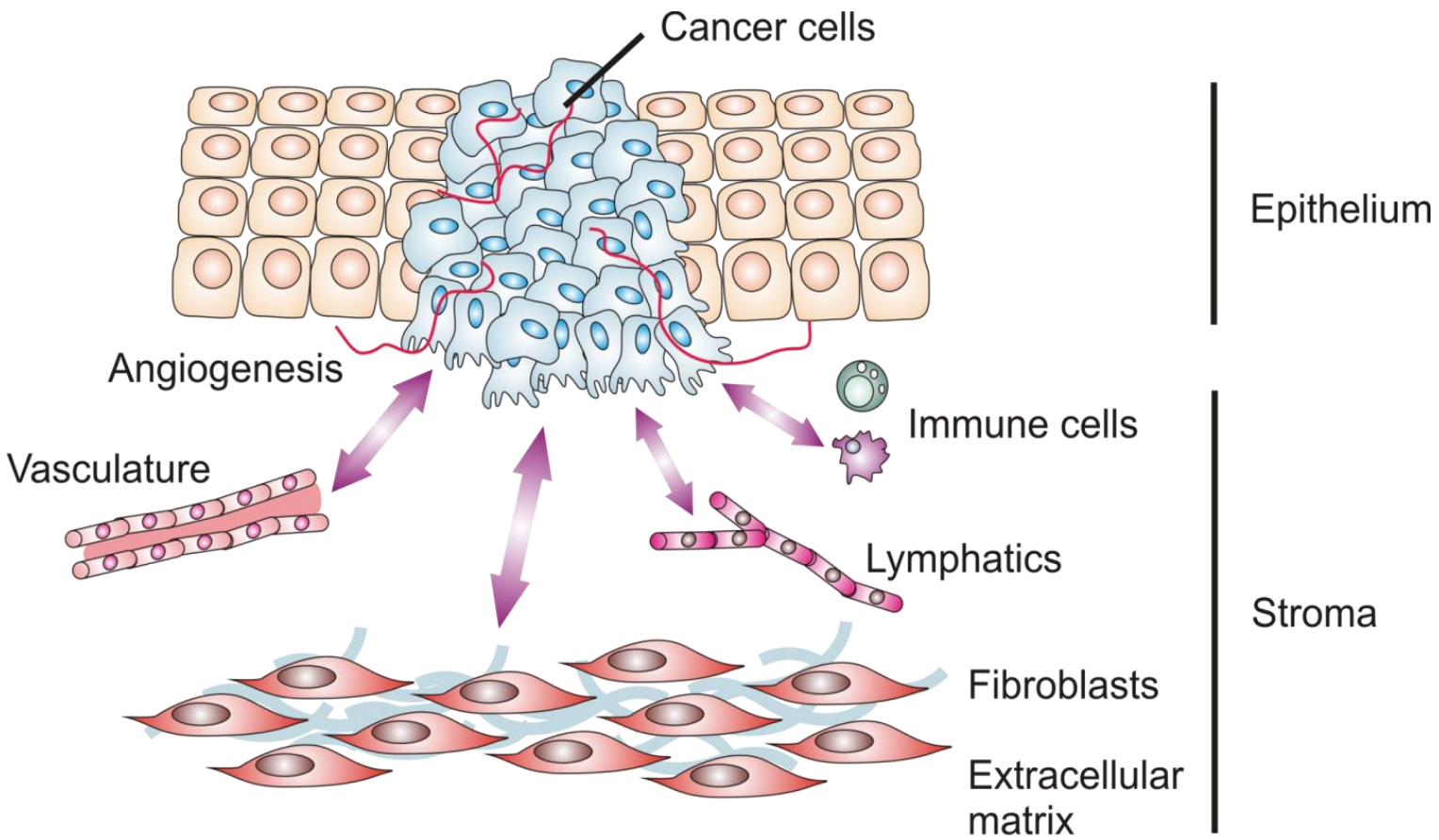


Figure 1.5 The tumour microenvironment: The development and progression of epithelial tumours is profoundly influenced by the surrounding microenvironment (the reactive stroma), composed of fibroblasts, blood vessels and lymphatics, extracellular matrix components and immune cells. Any or all of these can interact with the cancer cells and influence their behaviour (illustrated by purple arrows).

activation. This process creates a supportive tumour microenvironment for the carcinoma, contributing and promoting its progression (Stover *et al*, 2006). Normal stroma can however also play a limiting role in the progression of malignant cells, halting their growth as a result (Stover *et al*, 2006; Shekhar *et al*, 2001).

The processes of acute inflammation and repair consist of a strict pattern and course of events and when this ordering is altered during pathological conditions, including chronic unresolved inflammation and carcinogenesis, a disorganised microenvironment can be formed (Kumar *et al*, 2005). The formation and development of tumours has been described as 'wounds that do not heal' (Dvorak *et al*, 1986). In a normal scenario when epithelial cells proliferative at an unusually high rate, they trigger a chronic inflammatory reaction in order to re-establish homeostasis and repair the damaged tissue by remodelling it (Albini *et al*, 2005). Epithelial cells, fibroblasts, granulocytes and macrophages are all involved in recreating the correct homeostatic balance but they can in fact react in a contradictory manner and begin to promote the survival and increase the replication of the dysfunctional epithelial cells (Albini *et al*, 2005). Inflammatory angiogenesis can occur during this process and has been shown to play a role in cancer progression (Sparmann and Bar-Sagi, 2004; Albini *et al*, 2005).

In order for cancer cells to survive and thrive they generate their own supportive microenvironment. They release growth factors including basic growth factor (BGF), factors from the VEGF family, platelet derived growth factor (PDGF), EGFR ligands, interleukins, cytokines including tumour derived growth factor-β (TGF-β) and colony-stimulating factors. The growth factors that they release can act in a paracrine fashion to induce tumour-stromal interactions that can result in the promotion of angiogenesis and trigger inflammatory responses. The growth factors also activate surrounding cell types including fibroblasts, smooth-muscle cells and adipocytes. This activation can result in the additional release of additional growth factors and proteases, all of which are released into the surrounding microenvironment.

Cancer cells can signal via a paracrine mechanism, they can also act in an autocrine manner, acting on themselves to release proteolytic enzymes. The release of these enzymes can lead to the remodelling of the ECM and the basement membrane, promoting a pro-invasive and pro-migratory environment. The newly remodelled ECM can release MMPs which can result in the activation of cell surface receptors and ECM-bound growth factors. This process promotes the crosstalk that occurs within the tumour microenvironment including that between cancer cells and fibroblasts.

The communication network that exists within the tumour microenvironment is complex. Autocrine and paracrine signalling not only exists between tumour cells but also between cells found within the stroma. The communication network can also exist in reverse. In recent years studies have suggested that the microenvironment may not be caused by dysfunctional epithelial cells but may act as a factor in promoting their further dysfunction by promoting their invasive nature and their growth (Albini and Sporn, 2007). The paracrine control that the tumour microenvironment exerts on the epithelial cells that it surrounds can be altered depending on the cell types present (Albini and Sporn, 2007). Fibroblasts form the largest component of the tumour microenvironment and there are a number of different types of fibroblasts that have been identified in promoting tumourigenesis (Kalluri and Zeisberg, 2006).

1.3.1 Fibroblasts

Fibroblasts are the most numerous cell type found within the stroma. They are non-vascular, non-epithelial and non-inflammatory cells (Tarin and Croft, 1969). Fibroblasts play important roles in regulating the differentiation of epithelial cells, ECM deposition, inflammation and wound healing (Tomasek *et al*, 2002; Parsonage *et al*, 2005). Fibroblasts synthesise type I, type III and type V collagen and fibronectin (Rodemann and Muller, 1991; Tomasek *et al*, 2002), they secret type IV collagen and laminin, both of which are involved in the formation of the basement membrane and fibroblasts secret MMPs which highlight the importance of the cell type in maintaining ECM homeostasis (Wiseman and Werb, 2002). Fibroblasts also secrete growth factors and help to control direct mesenchymal-epithelial cell interactions between epithelial cells (Wiseman and Werb, 2002).

1.3.1.1 Cancer associated fibroblasts

Orimo *et al* (2005) have observed clear differences between normal fibroblasts and fibroblasts located in the stoma surrounding invasive human mammary carcinoma masses. The fibroblasts located within the tumour microenvironment enhanced the growth of the tumour, promoted increased vascularisation within the tumour, expressed more α-smooth muscle actin (α-SMA), produced increased levels of stomal cell-derived factor 1 (SDF-1) which recruited endothelial progenitor cells (EPCs) into the tumour and therefore caused an increase in angiogenic promotion (Orimo *et al*, 2005). The SDF-1 released from the fibroblasts can work via a paracrine mechanism and can act directly on the breast carcinoma cells by binding to the CXC chemokine receptor 4 (CXCR4) triggering its stimulation and activation (Orimo *et al*, 2005). It is not known if the altered fibroblasts are a result of the carcinoma cells altering the phenotype of the normal fibroblasts or if the myofibroblasts are recruited to the area from

outside the tumour microenvironment (Ishii *et al*, 2003). There is increasing evidence that the former suggestion is the most likely.

All fibroblasts found within the tumour stroma can be described as cancer associated fibroblasts (CAFs) (Anderberg and Pietras, 2009). This title is used to describe any fibroblasts found within the given area regardless of the biomarkers that they possess (Anderberg and Pietras, 2009). In a morphological sense CAFs are described as large cells that have an elongated spindle shape. There are different types of CAFs that vary in their phenotypic marker expression profiles. It is not clear if the differences between CAF types reflect in their function and ability to promote tumour progression. Due to their distinct characteristics and differences in comparison to normal fibroblasts, CAFs are thought to contribute to tumour growth and carcinogenesis. The term protomyofibroblasts has been used to describe the phenotypic spectrum of changes associated with CAFs (Desmouliere *et al*, 2003). The morphological changes associated with fibroblasts located in the tumour microenvironment can range from those that are found within the defined area and show no obvious phenotypic changes, through to those that express increased levels of specific markers including α-SMA. These CAFs are termed myofibroblasts.

1.3.1.2 Myofibroblasts

Myofibroblasts have been identified in the stroma surrounding invasive breast cancers (Sappino *et al*, 1988). Myofibroblasts are characterised by a high expression level of α-SMA and have distinct gene-expression profiles (Allinen *et al*, 2004). The increased expression of vimentin, desmin, fibroblast activation protein and MMPs including MMP-2 are all markers of myofibroblasts. Myofibroblasts found within the tumour microenvironment of a number of epithelial carcinomas are similar to that found at sites of wound healing (Serini and Gabbiani, 1999; Mueller and Fusenig, 2004). At the site of injury myofibroblasts are responsible for the production of SDF-1 and the subsequent recruitment of EPCs which lead to tissue regeneration and repair. Therefore their recruitment and activation in the tumour microenvironment may be as a result of their natural characteristics and could promote tumour progression as a consequence of their normal function. Cancer cells could utilize the body's normal stromal response to tissue injury and in the process create a tumour microenvironment with similar characteristics which includes increased levels of SDF-1, EPC recruitment and promote an inflammatory response which can in turn promote tumourigenesis (Orimo *et al*, 2005). Expression of CXCR4 on carcinoma cells correlates with poor prognosis and is also known to promote primary tumour growth (Staller *et al*, 2003; Balkwill, 2004). Increased SDF-1 production by myofibroblasts within the tumour microenvironment could increase CXCR4

expression via paracrine stimulation. The targeting of this pathway could be a possible therapeutic target in cancer treatment (Orimo *et al*, 2005).

The number of myofibroblasts is known to be elevated in many cancer types including skin, prostate and breast. Myofibroblasts are thought to contribute to tumour progression via a number of methods. They can release pro-migratory factors including ECM components, MMPs and serine proteases. They also release growth factors and cytokines including insulin-like growth factor-1 (IGF-1) and hepatocyte growth factor (HGF) which can both contribute to cell survival (Allen and Jones, 2009). IGF-1 can also stimulate tumour-cell migration and HGF can stimulate tumour-cell invasion. Factors including VEGF and monocyte chemotactic protein 1 (MCP1) which are also secreted by myofibroblasts can also contribute to the tumour microenvironment and stimulate further stromal-epithelial interactions and promote angiogenesis and the recruitment of inflammatory cells, all of which are processes that contribute to tumour progression (Allen and Jones, 2009).

Myofibroblasts promote angiogenesis via the release of SDF-1. SDF-1 is responsible for the recruitment of EPCs into the tumour. This recruitment increases angiogenesis and directly contributes to an increase in tumour mass as a result of interactions with the CXCR4 tumour cell (Allen and Jones, 2009). Myofibroblasts have the ability to promote angiogenesis and increase tumour mass in the absence of cancer cells suggesting that the function and characteristics of myofibroblasts are not solely reliant and in response to tumour-derived signals and growth factors (Allen and Jones, 2009). Other growth factors and signals found within the stroma that surrounds the myofibroblasts also contribute to their phenotype. Connective tissue growth factor (CTGF) is found in greater levels within myofibroblasts and is commonly potently stimulated by TGF-β. CTGF is found to promote angiogenesis and tumour growth.

1.3.1.2.1 Origin

The precise origin of 'CAFs' remains debateable. Myofibroblasts found within the tumour microenvironment can originate from different sites of origins and display common and site-specific properties depending on the environmental niche that they have originated from.

1.3.1.2.1.1 Stromal fibroblasts

The majority of myofibroblasts are thought to originate from the activation of stormal fibroblasts within their local environment. It is well known that the multifunctional TGF-β cytokine can trigger the transdifferentiation and chemotaxis of fibroblasts into myofibroblasts (Anderberg and Pietras, 2009). There are three TGF-β isoforms; TGF-β1, TGF-β2 and TGF-β3. The isoforms are initially present within a complex which contains latent TGF-β binding

proteins. These proteins are proteolytically removed in order to activate and release TGF-β. TGF-β exerts its effects by binding to the TGFβ2 receptor (TGFβ2R) resulting in its activation and the recruitment of the TGFβ1R and subsequent downstream cytoplasmic signalling through many different signalling cascades including the intracellular Smad proteins -2 and -3 and serine-threonine receptors (Border and Noble, 1994; Attisano and Wrana, 2002). The Smad proteins bind to Smad4 and translocate into the nucleus resulting in the activated transcription of target genes. TGF-β can also activate other signalling pathways including the ras/mitogen activated extracellular signal regulated kinase (MEK)/extracellular signal regulated kinase (ERK) pathway (Leask, 2010).

Early studies on fibroblasts identified that they were responsible not only for the mesenchymal regulation of germ layers that took place during organogenesis but were also able to regulate the proliferation and differentiation of epithelial tumours (Ronnov-Jessen *et al*, 1996; Cunha *et al*, 1985). Stroma that was identified as being transformed could induce malignancy in lung and mammary epithelial cells (Nakamura *et al*, 1997). Normal fibroblasts are also known to be capable of inducing a malignant phenotype in skin and prostate epithelium (Hayashi and Cunha, 1991; Cooper and Pinkus, 1977). Bhowmick *et al* (2004) identified that loss of TGF-β signalling in fibroblasts resulted in increased fibroblast and luminal epithelial proliferation. The reduction in signalling also resulted in an increase in the development of preneoplastic lesions within the prostate epithelial. In TGFβ2R knockout mice levels of c-Myc were increased and the expression of cyclin dependent kinase (CDK) inhibitors, p21 and p27 were reduced. Phosphorylation of HGF receptor was also increased in the knockout mice and was suggested to be the cause for increased stimulation of epithelial proliferation observed. TGF-β signalling in fibroblasts has the ability to modulate the growth and oncogenic potential of adjacent epithelial cells in the surrounding environment.

1.3.1.2.1.2 Tumour cells

It has also been suggested that myofibroblasts could also originate from epithelial and endothelial tumour cells and develop into myofibroblasts through EMT. This may be the reason why similar genetic alterations have been observed within the tumour epithelium and its surrounding stroma (Anderberg and Pietras, 2009). Other mesenchymal cells including vascular smooth muscle cells, pericytes or adipocytes have also been suggested as possible cell types which myofibroblasts could originate from. Experimental evidence for this theory however is largely lacking (Anderberg and Pietras, 2009).

1.3.1.2.1.3 Bone marrow-derived cells

Bone marrow-derived cells that were genetically marked have shown that they too can contribute to myofibroblasts populations found within tumour-associated stroma (Anderberg and Pietras, 2009). This is also true for mesenchymal stem cells.

1.3.1.2.1.4 Genetic alterations

Fibroblasts surrounding breast cancer cells showed altered genetic characteristics including somatic mutations and a LOH (Moinfar *et al*, 2000; Kurose *et al*, 2002; Fukino *et al*, 2004). The genetic alterations could be responsible for the change in fibroblast phenotype resulting in the enhancement of their tumourgenetic qualities. Fibroblasts may exhibit epigenetic alterations which could promote an activated autocrine TGF-β feedback loop (Ronnov-Jessen *et al*, 1996; Serini and Gabiani, 1999), which may result in normal fibroblasts differentiating into myofibroblasts. The stroma that surrounds tumours may be genetically unstable and may undergo changes including LOH that results in changes in cell types and the stimulation and production of myofibroblasts. Somatic *TP54* genetic mutations have been observed in breast tumour epithelium and stroma and have been linked to lymph node metastases (Moinfar *et al*, 2000; Kurose *et al*, 2002; Fukino *et al*, 2004). Questions relating to the preparations of tissue within this study suggest that the results might not be conclusive (Anderberg and Pietras, 2009). Another study that is more extensive has suggested that genetic mutations within myofibroblasts is a very rare event and can therefore not be used to explain the origin of all myofibroblasts (Anderberg and Pietras, 2009). These studies highlight the controversial beliefs surrounding this area of research.

In the absence of tumour cells, the phenotype of myofibroblasts could be created and maintained by epigenetic modulation of the cell's DNA. Genetic alterations have frequently been associated with cancer progression, in particular the role that genetic mutation in oncogenes and tumour suppressor genes, and chromosomal abnormalities and instabilities play in tumour initiation and development (Baylin and Ohm, 2006; Hahn *et al*, 1999; Hanahan and Weinberg, 2000; Kinzler and Vogelstein, 1997). Research conducted however has also identified a role for epigenetic mutations in tumour progression. Epigenetics is a term used to describe the alterations in gene expression which are mediated by mechanisms that do not affect or alter the primary DNA sequence. This line of research has been associated with not only determining and altering cancer cell phenotype but also that of cells found within the tumour microenvironment including fibroblasts and may therefore provide a possible explaination for the origin of myofibroblasts. Epigenetic changes can include the loss or gain of DNA methylation and histone modification (Baylin and Ohm, 2006; Jones and Laird, 1999; Hermann and Baylin, 2003; Jones and Baylin, 2002).

In breast cancer and colorectal cancer DNA methylation has been identified in fibroblasts that have been isolated from the tumours. The patterns of epigenetic mutations have also been partnered with changes in mRNA levels (Allen and Jones, 2009). The genetic variability of the host has also been suggested as another potential source of origin for myofibroblasts (Allen and Jones, 2009). Non-tumour fibroblasts isolated from women who have been diagnosed with breast cancer have been identified as differing from fibroblasts isolated from women who don't have breast cancer. This identification could be a result of single nucleotide polymorphisms (SNPs) which are genetic mutations that occur within many genes including MMP enzymes which are responsible for modifying the extracellular matrix (Allen and Jones, 2009).

1.3.1.3 Fibrocytes

Fibrocytes are another cell type found within the tumour microenvironment. They are a unique population of CD45+ cells that are distinct from endothelial cells, epithelial cells, fibroblasts, monocytes, dendritic cells, T lymphocytes and Langerhans cells that were originally identified in the wound chamber of an experimental wound healing model (Keeley *et al*, 2010). They are spindle-shaped and are morphologically distinct from leukocytes in that they display prominent cell surface projections which can be seen when the cells are scanned with an electron microscope. Fibrocytes can be identified by their distinct set of characteristics which change during their development. They account for 0.1-1% of all nucleated cells found within the peripheral blood found with the body of a healthy individual (Keeley *et al*, 2010). Fibrocytes can express collagen, procollagen and CD34 at a site of injury. They respond to the injury and appear at the site of injury much more quickly than fibroblasts do, usually within one day of the injury occurring. Their ability to appear so quickly at the site has led to suggestions that they must originate from within circulation (Keeley *et al*, 2010). They help to promote angiogenesis and release chemokines, cytokines and growth factors that induce fibroblast hyperplasia. They can also contribute and release secretion factors of the extracellular matrix. TGF-β and endothelin-1 (ET-1) can stimulate fibrocytes to differentiate into myofibroblasts (Keeley *et al*, 2010). When fibrocytes are cultured for lengthy periods of time they can acquire an increase in α-SMA expression and can begin to lose the expression of CD34 and CD45 (Keeley *et al*, 2010). These processes are dependent on the physical environment that surrounds the fibrocyte. Their similarities to myofibroblasts make them an important target to study when investigating the role the tumour microenvironment plays in cancer progression.

1.3.1.4 Cancer stem cells

Stem cells are another cell type associated and found within the tumour microenvironment (Clarke *et al*, 2006). They have been identified as being fundamental in the development and maintenance of several forms of human cancer (Jordan *et al*, 2006; Wang and Dick, 2005; Singh *et al*, 2004; Reya *et al*, 2001). A cancer stem cell can be described as a cell found within a tumour that possess the ability to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumour (Clare *et al*, 2006).

There are a number of theories regarding the role that cancer stem cells play in initiating tumour development and progression. It has been suggested that tumours can derive from organ stem cells that retain self-renewal properties and that over time these cells can obtain epigenetic and genetic changes that are required for tumorigenicity (Clarke *et al*, 2006). It has also been hypothesised that tumour stem cells are proliferative progenitors that acquire self-renewal capacity (Clarke *et al*, 2006).

The ability of stem cells to enter the cell cycle infrequently is a characteristic which may allow cancer stem cells to form a small population of cells that are intrinsically resistant to many therapeutic agents which target and kill proliferating cells (Clarke *et al*, 2006).

1.3.2 Contributions of cancer associated fibroblasts to tumour progression

CAFs contribute to cancer progression via a number of different mechanisms:

1.3.2.1 Growth signals

Myofibroblasts release many growth factors and hormones including HGF, EGFR, fibroblast growth factor (FGF) and Wnt families and cytokines including stromal derived factor-1α (SDF-1α) and interleukin (IL)-6. These factors can act via an autocrine mechanism on the myofibroblasts themselves to further promote their growth and development and the factors can also act on the surrounding epithelial and endothelial cells resulting in the stimulation of phenotype transformation; an example of the tumour-stromal interactions that happen within the tumour microenvironment (Pietra and Ostman, 2010).

1.3.2.2 Evasion of apoptosis

Myofibroblasts produce IGF -1 and -2 and increase the expression of survival signals within the tumour cells. This allows them to avoid cellular apoptosis and stimulates their growth. Myofibroblasts also produce lots of ECM components including large quantities of collagen used to enhance cross linking and increase the invasive nature of tumours and aid malignant growth. The phosphoinositide 3 (PI3)-kinase and Akt survival pathways are also triggered by

myofibroblasts in response to increased integrin ligation by collagen fibres which promote downstream signalling.

1.3.2.3 Sustained angiogenesis

Myofibroblasts trigger the release of pro-angiogenic factors into the tumour microenvironment. Many pro-angiogenic factors including members of the VEGF family are produced at greater levels in tumours in comparison to normal tissue counterparts. A greater induction in the activity of the VEGF promoter is also observed. Myofibroblasts can also increase VEGF production by the activation of the bradykinin (BK) 2 receptor (B2R) resulting in an increase in prostaglandin E2 (PGE_2) release. Via a paracrine signalling mechanism myofibroblasts can stimulate PDGF receptor signalling. Using an autocrine signalling mechanism they can also cause cytokine CXCL14 to induce the expression of basic fibroblast growth factor (BFGF)-2. The cells can also secret SDF-1 which increases the recruitment of endothelial progenitor cells into the tumour neo-vasculature, promoting angiogenesis.

1.3.2.4 Tissue invasion and metastasis

In order for tumours to become more invasive and be able to metastasise myofibroblasts promote the EMT of tumour cells. They achieve this by secreting TGF-β and HGF. Myofibroblasts also act as a source of increased protease activity including increased MMP production, cathespins and plasminogen activators, all of which help to remodel the ECM. The proteases weaken the connections between the tumour cells and adjacent cells and the underlying basement membrane. The proteases degrade the ECM and provide the environment with additional growth factors and ECM-adhering properties. Epithelial cells can undergo pro-invasive changes when exposed to stromal cells.

1.3.3 Signalling networks within the tumour microenvironment

Complex signalling pathways and networks occur within the microenvironment in order for cells to be able to perform basic activities and actions. The ability of cells to control and govern these communication systems allows them to perceive and correctly respond to their microenvironment. This is vital and allows control of basic processes including development, tissue repair, and immunity as well as normal tissue homeostasis. Errors that occur within this communication network can result in the formation of diseases including autoimmunity, diabetes and cancer. This thesis focuses on trying to determine the signalling networks associated with head and neck cancer pathogenesis.

1.3.3.1 Intracellular signalling and extracellular signalling

Cellular signalling can be classified as intracellular and extracellular. Cross over between the two mechanisms and the cellular components involved in the processes exist. Intracellular signalling is used to describe pathways which are responsible for transmitting information within a cell. This information is usually received via an external stimulus or from signals that are generated from within the cell. Extracellular signalling is used to describe the process in which a cell synthesises and releases a signalling molecule into the surrounding microenvironment. This signal molecule can then be transported to another cell onto which it is detected by a specific receptor protein. This event results in the formation of a receptor-signal complex on the surface of the cell which ultimately promotes a signal that can alter the cells metabolism, function, or development. The removal of the signal from the receptor-signal complex usually terminates the cellular response. There are a number of different factors and cellular machinery that can be involved in this series of events.

The EGFR is a receptor tyrosine kinase and is upregulated in a number of malignancies including HNSCC (Rubin *et al*, 1998). It is 175 kDa in size and an increase in the expression of this receptor in HNSCC correlates with a poor prognosis (Rubin *et al*, 1998). Activation of the receptor via ligand induced autophosphorylation, can lead to downstream signalling including that of the mutagenic Raps/Raff/ERK 1/2, p38 MAPK and PI3-K/AKT pathways, all of which are involved in regulating cell proliferation, growth and differentiation (Cai *et al*, 2010; Ciardiello and Tortora, 2008; Avraham and Yarden, 2011). Grandis *et al* (1998) have demonstrated that by targeting the EGFR, the proliferation and invasion of HNSCC can be reduced. The EGFR has been blocked as a possible drug therapy target in the treatment of HNSCC and antitumour effects have been observed when EGFR blockade has been combined with radiation treatment (Bonner *et al*, 2006).

The EGFR pathway can be triggered by a number of G protein-coupled receptor (GPCR) ligands including lysophosphatidic acid (LPA), gastrin-releasing peptide (GRP), thrombin, ET-1, BK and angiotensin II (Ang II) (Lui *et al*, 2003; Gschwind *et al*, 2003), an example of extracellular signalling. The process via which GPCRs activates the EGFR with an increase in activation of protein kinase C (PKC), an elevation in intracellular Ca^{2+} and the generation of reactive oxygen species (ROS) (Liebmann, 2011; Higuchi *et al*, 2007). GPCRs are known to promote tumour cell growth upon their activation in a number of different cancer types, including HNSCC. Therefore a number of drug therapies have targeted GPCRs as a possible treatment method for a number of cancers (Li *et al*, 2005). GPCRs make good, possible targets because they allow specific inhibition and are often over expressed in a number of different tumours.

It is widely believed that upstream kinases including src, a tyrosine-specific protein kinase encoded by the v-src oncogene of RSV, and metalloproteases and/or ADAM-dependent shedding of cell-surface bound EGF-like ligands can also transactivate the EGFR (Higuchi et al, 2007). This explains how GPCR ligands can activate the EGFR via an autocrine mechanism which involves the release of EGFR ligands including transforming growth factor-α (TGF-α), amphiregulin and heparin bound-EGF (HB-EGF) (Lui *et al*, 2003; Pai *et al*, 2002), another example of extracellular signalling stimulation. This result in GPCR activation triggers intracellular signalling via ADAM metalloproteinase-dependent transactivation of the EGFR. In breast cancer, BK is known to act as an inflammatory molecule that has the ability to activate EGFR leading to an increase in proliferation of breast cancer cells (Greco *et al*, 2005). PGE_2 has also been identified as another inflammatory molecule that can activate EGFR leading to the activation of Src and a number of MMPs (Pai *et al*, 2002; Buchanan *et al*, 2003). This activity has been observed in colon cancer and is an example of how the intracellular and extracellular signalling pathways can overlap. Both BK and PGE_2 are GPCR ligands and targeting both these receptors along with the EGFR could be a possible future target therapy in the treatment of cancer and could create enhanced antitumour effects in comparison to just targeting the EGFR alone (Thomas *et al*, 2006).

Prostaglandins and thromboxanes are important in the regulation of cellular growth and under pathological conditions, including inflammation and tumour growth, their expression levels can be altered and therefore the pathways that they regulate can be modified. Cyclooxygenases (COXs) are responsible for the conversion of arachidonic acid into PGEs and thromboxanes. Cyclooxygenase-2 (COX-2) is mitogen induced. Its expression is elevated in lung cancer in comparison to non-malignant tissues (Castelao *et al*, 2003; Hida *et al*, 1998). An increase in the expression levels of COX-2 leads to an increase in the production of PGE_2. PGE_2 and thromboxane A2 are procarcinogenic and can both promote new vessel formation, angiogenesis and increased tumour growth (Ermert *et al*, 2003). Prostacyclin has the opposite effects and can act as a potent vasodilator and has the ability to inhibit cellular growth. These components are all involved in the processes associated with intracellular signalling. It has been demonstrated that inhibition of COX-2 using inhibitors that target it results in reduced proliferation of malignant cells *in vitro* (Hida *et al*, 1998) and a reduction in the growth of tumours and their metastatic potential (Grubbs *et al*, 2000).

The array of proteins found within the ECM provide both physical support to the structure of the cell and also stimulate other biological functions including promoting interactions between a range of factors including adhesion molecules, growth factors, signal receptors and themselves. The ECM can act as a reservoir for growth factors including VEGF and BFGF (Kim

et al, 2011). Signalling within the ECM can involve integrins. Integrins are heterodimeric transmembrane receptors and consist of 18 α subunits and 8 β subunits which can form 24 different non-covalent assemblies. This allows them to interact with a number of components of the ECM which results in the production of both mechanical and chemical signals between themselves and the actin cytoskeleton. They can stimulate signalling independently or can bind with other growth factor receptors including IGF-1, VEGF receptor, EGFR, PDGF receptor and the TGF-β receptor and synergistically they can activate signalling which can affect proliferation, migration, differentiation and apoptosis (Kim *et al*, 2011; Nelson and Bissell, 2005). Other ECM proteins including tenascin-C and fibronectin are consistently upregulated in cancer and can promote extracellular signalling within the tumour microenvironment (Allen and Jones, 2011).

MMPs have been implicated in the remodelling of the ECM. The microenvironment provides a large source of MMPs which are all catalytically activated from a latent pro-MMP form. This activation is achieved by the removal of the propeptide domain by proteolytic cleavage. Membrane type-1 (MTI)-MMP is responsible for the activation of other MMPs including pro-MMP-2 and -13. MMPs are capable of degrading proteins within the ECM and have therefore been implicated in the promotion of tumour invasion and metastasis. MMPs can however also process a number of bioactive molecules and are responsible for their release into the surrounding microenvironment. MMPs can cleave cell surface receptors, apoptotic ligands including the FAS ligand, chemokines and cytokines (Nelson and Bissell, 2005).

MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs) which play a vital role in balancing the proteolytic activity associated with tumours, therefore reducing their invasive and metastatic ability. This is an example of a process which is in place to control extracellular signalling mechanisms. The TIMPs bind to the zinc binding catalytic site on the MMPs in a 1:1 ratio. There are four types of TIMPs, TIMPs-1 -2, -3 and -4. TIMP-1 can inhibit nearly all MMPs and TIMPs-2, -3 and -4 can inhibit all MMP activities. TIMP-3 is an extracellular matrix protein and has the potential ability to inhibit a number of ADAMs enzymes. MMP-dependent proteolysis is responsible for the control of activated levels of MMPs and TIMPs (Baker *et al*, 2002).

Urokinase plasminogen activator (uPA) is a 54 kDa serine protease (Andreasen *et al*, 1997). Tumour cells secrete the proteinase in a pro form. The pro form of uPA, pro-uPA, binds to its specific receptor on the surface of the tumour cell and is activated by cathepsin B. After the proteinase has been activated it can convert the proenzyme plasminogen into the catalytically active plasmin. Plasmin has the ability to degrade components found within the stroma of the

tumour resulting in the modification of the tumour microenvironment. uPA is also a constituent of the cascade involved in the activation of metalloproteinases. There are two inhibitors of uPA, plasminogen activator inhibitor (PAI)-1 and -2, which function by causing the internalization of the uPA ternary complex (Duffy, 1993; Foekens *et al*, 2000; Chambers *et al*, 1995; Sier *et al*, 1998).

The ADAMs group of enzymes are responsible for regulating cell to cell and cell to ECM interactions that occur within the microenvironment. The family of enzymes have been linked with the process of proteolytic shedding of proteins from the surface of cells. This results in the rapid transformation of key signalling pathways (Murphy, 2008). The ADAMs family of enzymes are all multi-domain type 1 transmembrane proteins. Some members of the family, including ADAM12 lack the transmembrane and cytoplasmic regions of the structures. About half of the family has proteolytic activity and are responsible for modulating the activity of adhesion molecules, membrane cytokines and growth factors and the receptors that they exert their effects through (Murphy, 2008). The enzymes can cleave these transmembrane proteins allowing their solubilisation and release into the surrounding environment. The upregulation of ADAMs enzymes has been implicated in a number of cancers including ADAM17. ADAM17 has proteolytic activity and its structure consists of metalloprotease and disintegrin domains and a cytoplasmic domain that is often rich in SH3-binding sites (Seals and Courtneidge, 2003; Blobel, 2005). It is involved in regulating the release of a number of proteins, including TGF-α, amphirgulin and HB-EGF, from the surface of cells which are know upon release to act as ligands for the EGFR. Aberrant regulation of this receptor is known to occur in a number of tumours and is therefore a common drug target. The expression of ADAMs enzymes has also been identified at the invasive front of some tumours. The stroma surrounding carcinomas has been implicated as a possible source for ADAMs.

The release of soluble factors including the mitogenic peptides BK, ET-1, angiotensin II (Ang II), substance p, neurotenisn and GRP and cytokines including TGF-β, via intracellular and extracellular signalling mechanisms, into the surrounding microenvironment can stimulate autocrine and paracrine signalling within the reactive stroma. The processes can encourage and support crosstalk between different cell types. These signalling mechanisms are necessary for the maintenance of a normal physiological environment but dysregulation of the pathways can result in the enhancement of signalling which can be associated with disease progression.

1.4 Mitogenic peptides

Bradykinin (BK), endothelin-1 (ET-1) and angiotensin II (Ang II) are all examples of small regulatory peptides that have been implicated in the progression of a number of malignancies.

They often act as ligands for specific G-protein coupled receptors (GPCRs). As mentioned before the activation of GPCRs by mitogenic peptides can trigger many intracellular and extracellular signalling pathways. Alteration of these signalling pathways can lead to deregulated cell behaviour and function.

1.4.1 Bradykinin

Bradykinin (BK) is an example of an important mediator of a wide variety of physiological and pathophysiological responses including pain, inflammation, cell division, mitogenicity and sperm motility (Bhoola *et al*, 1992. Hall, 1997). BK is produced from protein kininogens that are situated within plasma and tissue (Stephan *et al*, 2003). BK exerts its effects through the B1 receptor (B1R) and B2 receptor (B2R) kinin receptors which are both GPCRs. Upon their activation they can stimulate ERK 1 and 2 and mitogen activated protein kinases (MAPKs) via distinct pathways. The activation of these pathways results in the release of PKC and the activation of EGFR (Adomeit, 1999), tyrosine kinases focal adhesion kinase and c-Src (Velarde, 1999).

BK and its receptors have been implicated in the progression of a number of cancers and a number of studies have investigated the involvement of the peptide in HNSCC. BK can promote tumour progression in HNSCC by promoting cell proliferation and invasion (Thomas *et al*, 2006). BK in this instance exerts its effects through both B1R and B2R. Changes in the expression of each receptor can vary between cancer types. In malignant prostate cancer an up-regulation of B1R is observed (Taub *et al*, 2003) whereas in human gliomas an increase in the expression of B2R was seen (Zhao *et al*, 2005). B2R is also overexpressed in HNSCC in comparison to normal oral mucosa suggesting that the receptor may contribute to the progression of the disease (Zhang *et al*, 2008). BK has been shown to increase the levels of COX-2 in HNSCC (Zhang *et al*, 2008). Under basal conditions COX-2 is absent or present at very low levels within the oral mucosa (Vane *et al*, 1998). BK causes an increase in COX-2 mRNA levels as a result of the peptide activating the EGFR. This activation process triggers downstream signalling resulting in the activation of the MAPK pathway which results in an increase in COX-2 along with an increase in PGE_2 production and release (Zhang *et al*, 2008). PGE_2 and BK can both activate the EGFR in HNSCC and can cause an increase in cell proliferation and invasion (Thomas *et al*, 2006). Both GPCR ligands can activate MAPK via an EGFR-dependent and an EGFR-independent mechanism (Thomas *et al*, 2006). This process was inhibited when neutralising antibodies to TGF-α but not neutralising antibodies to HB-EGF or amphiregulin were used, suggesting that TGF-α is released from the cell surface of HNSCC

cells in response to PGE_2 and BK (Thomas *et al*, 2006). Further experiments conducted by Thomas *et al* (2006) showed that both the Src family kinases and ADAMs enzymes were involved in the signalling pathways activated by the treatment of HNSCC cells with PGE_2 and BK. The inhibition of the Src family kinases reduced MAPK phosphorylation and the siRNA knockdown of ADAM17 resulted in lower levels of TNF-α being released into the surrounding supernatant upon stimulation of HNSCC cells with BK (Thomas *et al*, 2006). This shows that BK causes MMP mediated release of TGF-α which can then act as a ligand to activate EGFR in an autocrine mechanism (Thomas *et al*, 2006).

1.4.2 Neurotensin

Neurotensin is a neuropeptide, 13 amino acids in size (Carraway and Leeman, 1978) and located within the central nervous system and in jejunal and ileal mucosa, both of which contain endocrine cells (Evers *et al*, 1990; Evers *et al*, 1992). Neurotensin exerts its effects by binding to the neurotensin receptor 1 (NTSR1) and neurotensin receptor 2 (NTSR2), both of which are GPCRs (Vincent *et al*, 1999). NTSR1 shows the highest affinity for neurotensin (Vincent *et al*, 1999). Upon binding to the NTSR1, neurotensin can trigger intracellular signalling including MAPK and NF-κB which both promote inflammatory responses and induces the secretion of neutrophil chemoattractants including IL-8 (Zhao *et al*, 2003; Zhao *et al*, 2005).

Neurotensin and NTSR1 have been implicated in a number of cancers including prostate, colon, lung and pancreatic (Thomas *et al*, 2003). It is thought that the neuropeptide stimulates the expression of tumour-promoting genes through the activation of its receptor and the triggering of signalling pathways, including those associated with intracellular Ca^{2+}, and the activation of transcription factors (Ehlers *et al*, 2000; Zhao *et al*, 2003; Leyton *et al*, 2002). Shimizu *et al* (2008) also identified that the expression of neurotensin and NTSR1 mRNA were elevated in HNSCC samples and was associated with metastatic spread and HNSCC progression. Their increased expression also resulted in an increase in IL-8 and MMP-1 transcripts within the HNSCC clinical samples (Shimizu *et al*, 2008). Neurotensin could also contribute to cancer progression by the sustained proinflammatory response that it can trigger (Law *et al*, 2012).

1.4.3 Substance P

Substance P is an undecapeptide. It belongs to a family of peptides called the tachykinin family. Substance P exerts its effects by binding to the neurokinin 1 receptor (NK-1R) (Hökfelt

et al, 2001). It plays a role in regulating the cardiovascular system, respiratory mechanisms, sensory perception, the control of movement, gastric motility, salivation and neuronal survival and degradation (Quartara and Maggi, 1998). Substance P is also described as a neuropeptide and has been associated with the processes of inflammation, pain and depression (Kramer *et al*, 1998; Harrison and Geppetti, 2001; Bang *et al*, 2003). Within the process of inflammation, substance P has been linked to the development and progression of mucosal inflammation (Koon and Pothoulakis, 2006).

Substance P has also been implicated in tumour progression and is thought to act as a mitogenic peptide via the NK-1R. The peptide is over expressed in a number of cancer cell lines including those isolated from neuroblastoma, glioma, melanoma and laryngeal cancers (Muñoz *et al*, 2004; Muñoz *et al*, 2008). Brener *et al* (2009) identified for the first time the presence of substance P and the NK-1R within HNSCCs. The binding of substance P to its receptor is known to trigger the MAPK cascade and induce the activation of ERK 1 and 2 which results in increased cellular proliferation and cell apoptosis resistance (Koon *et al*, 2004). Substance P can also act as a ligand for the EGFR resulting in its activation and stimulating cell proliferation as a consequence (Weinstock *et al*, 1998).

Little is known of the role of the mitogenic peptides, ET-1 and Ang II in HNSCC progression.

1.4.4 Endothelin-1

Endothelin-1 (ET-1) is a mitogenic peptide which acts primarily as a potent endogenous vasoconstrictor within the systemic and pulmonary circulatory systems (Yanagisawa *et al*, 1988). It also has various physiological roles including the maintenance of basal vascular tone, central respiratory regulation and renal homeostasis and is secreted mainly by endothelial and epithelial cells (Yanagisawa *et al*, 1988). There are three members of the ET family: ET-1, ET-2 and ET-3. Each peptide is made up of 21 amino acids (Inoue *et al*, 1989). ET-1 and ET-2 have similar structures. ET-3 however has a different structure. It consists of only 15 amino acids that are present in ET-1 and ET-2 plus six additional amino acids (Levin, 1995; Goldie, 1999; Walden *et al*, 1998; Masaki, 2000). The expression levels of the three different ETs vary in different cell types. ET-1 is the most abundant ET and is found mainly in endothelial cells but is also present in epithelial cells and fibroblasts, ET-2 in the kidneys and the intestine and ET-3 is primarily found within the brain (Levin, 1995). The different localisation of the three ETs could reflect their different functional abilities (Nelson *et al*, 2003). The presence of the different ETs results in a number of actions, which are mediated by the binding of ET to two cell surface GPCRs, ET_AR and ET_BR (Arai *et al*, 1990; Sakurai *et al*, 1992). The receptors are divided by the

different affinities that they have for the three different members of the ET family. ET_AR is highly specific for ET-1, whereas ET_BR has equal affinity for ET-1, ET-2 and ET-3 (Rubanyi and Polokoff, 1994). Each receptor has a different carboxy- terminal sequence and therefore generates divergent intracellular signals which influence tissue differentiation, growth and repair (Goldie, 1999). The ET_AR upon activation couples to heterotrimeric Gα proteins including Gαq, Gαs, Gα12/13 (Horstmeyer *et al*, 1996; Takigawa *et al*, 1995; Gohla *et al*, 1999) and in some cell types Gαi (Hilal-Dandan *et al*, 1994; Cadwallader *et al*, 1997). The ET_BR couples to Gαi and Gαq (Takigawa *et al*, 1995). The binding of any of the three ET family members to ET_AR or ET_BR results in their activation and a change in concentration of various secondary messengers. These include Ca^{2+}, 1,2-diacylglycerol (DAG), IP_3, arachiodonic acid and cyclic adenosine monophosphate (cAMP) (Horstmeyer *et al*, 1996). ET-1 can act as a mediator of many cardiovascular and renal disorders and has recently been implicated in acting as a progression factor in many tumour types. Antagonists designed to the molecule and their receptors have been tested as an anticancer therapy (Bagnato and Catt, 1998; Battistini *et al*, 1993; Pirtskhalaishvili and Nelson, 2000, Nelson *et al*, 2003).

1.4.4.1 Regulation of ET-1: The ET-axis

Endothelin converting enzyme (ECE) is a type 2 membrane bound zinc metalloprotease and is a member of the neutral endopeptidase family. ECE has a large extracellular or luminal C-terminal catalytic domain and a small N-terminal cytoplasmic domain (Lambert *et al*. 2008). There are two ECE genes, ECE-1 and ECE-2, both of which are part of the M13 peptidase family (Dawson *et al*, 2006). ECE-1 exists and is expressed as a covalent dimer (Schmidt *et al*, 1994; Takahashi *et al*, 1995; Shimada *et al*, 1996). It is present and active at both the cell surface and within intracellular organelles. ECE-1 is distributed in a wider range of tissues throughout the body and at a higher expression level compared to ECE-2 (Schmidt *et al*, 1994; Shimada *et al*, 1994; Schmidt-Ott *et al*, 1998; Emoto and Yanagisawa, 1995). ECE-1 is expressed in the endothelium of all organs and is also expressed within nonvascular cells including within the tissues of the brain and within secretory granules in neuroendocrine cells (Xu *et al*, 1994; Takahashi *et al*, 1995; Schmidt-Ott *et al*, 1998; Korth *et al*, 1999). The cellular distribution of

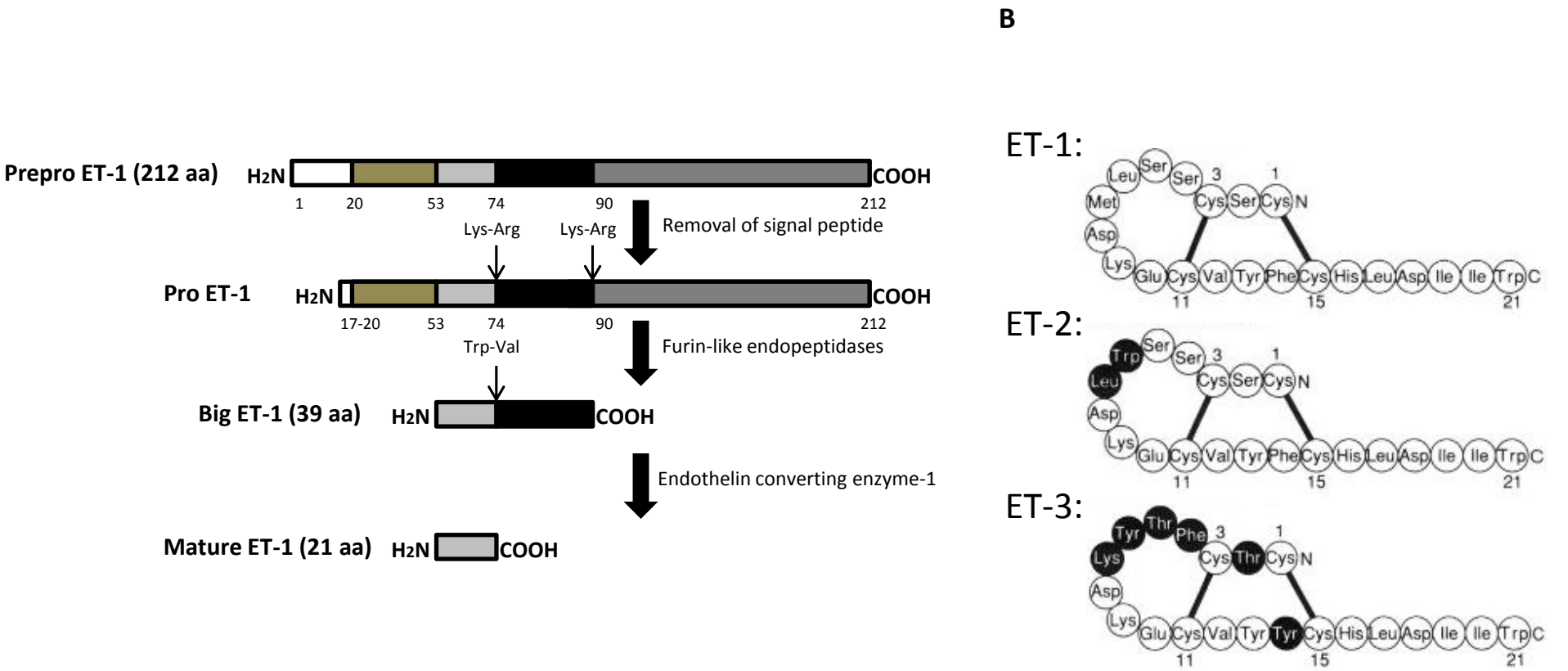


Figure 1.6 The biosynthesis of ET-1: The biosynthesis of ET-1 begins with the cleavage of prepro ET-1 to pro ET-1, followed by the cleavage of pro ET-1 to big ET-1 by a furin-like endopeptidase. The cleavage of big ET-1 to ET-1 is catalysed by endothelin converting enzyme-1 (ECE-1); a membrane bound metalloproteinase produces biologically active ET-1. This final proteolytic cleavage by ECE-1 occurs between the Trp-21-Val/Ile-22 bond **(A)**. There are three member of the ET family **(B)** (Figure adapted from Grant *et al*, 2003).

ECE is important in determining the biosynthetic pathway and production of specific ETs and the ability of the enzyme to synthesise and hydrolyse other peptides including BK, neurotensin and substance P (Nelson *et al*, 2003).

ECE-1 is responsible for the proteolytic cleavage of big-ET-1 to ET-1 between the Trp-21-Val/Ile-22 bond (Rubanyi and Polokoff, 1994) (Figure 1.6). The reaction can take place within the extracellular medium and in the secretory pathway (Xu *et al*, 1994; Harrison *et al*, 1995; Parnot *et al*, 1997). It is an essential step in the activation of ET-1 because its precursors have no biological activity (Figure 1.6) (Rubanyi and Polokoff, 1994).

ECE-1 has four distinct isoforms: ECE-1a, ECE-1b, ECE-1c and ECE-1d. All four isoforms are derived from a single gene (Figure 1.7). This is achieved through the use of alternative promoter regions for each isoform that direct expression of specific exons. The four isoforms of ECE are located within the secretory pathway and within the endosomal system. All four isoforms can exist as heterodimers and it has been suggested that this ability to heterodimerize may be responsible for the different subcellular locations of the different isoforms, again highlighting the importance that the location of ECE-1 plays in its function. ECE-1c is the most abundant isoform. It is widely distributed throughout the plasma membrane (Muller *et al*, 2003). The expression of ECE-1c is significantly elevated in tumours (Lambert *et al*, 2008). It is present within the primary malignant stromal cells of the tumour but is absent in benign stromal cells. The increase of ECE-1c in malignant stromal cells is thought to activate ET-1 and lead to a paracrine influence on the growth of the cells. ECE-1c is situated close to the inactive form of ET-1, big ET and therefore when ECE-1c is present it allows ET-1 to be readily activated. It is this activation of ET-1 that increases prostate cancer invasion and metastasis (Dawson *et al*, 2006).

Neprilysin (NEP) is an endopeptidase (Kerr and Kenny, 1974). It is 90-110 kDa in size and is localised at the cell surface (Sumitomo *et al*, 2000). It has the ability to inactivate a number of peptides that are physiologically active. NEP has the ability to inactivate a wide variety of physiologically active neuropeptides including ET-1, BK, neurotensin, atrial natriuretic factor, substance P, oxytocin, bombesin, bombesin-like peptides and Leu- and Met-enkephalins (Shipp *et al*, 1991; Shipp and Look, 1993). This results in a reduction in the local concentration of the specific peptides therefore reducing the level of receptor binding and signal transduction. The loss of this NEP activity has been implicated in a number of malignancies including small cell lung cancer, prostate cancer and lung cancer (Papandreou *et al*, 1998). The loss of activity of the surface endopeptidase results in the increased concentration of mitogenic peptides at the cell surface that the enzyme is normally responsible for inactivating. The inactivation of these

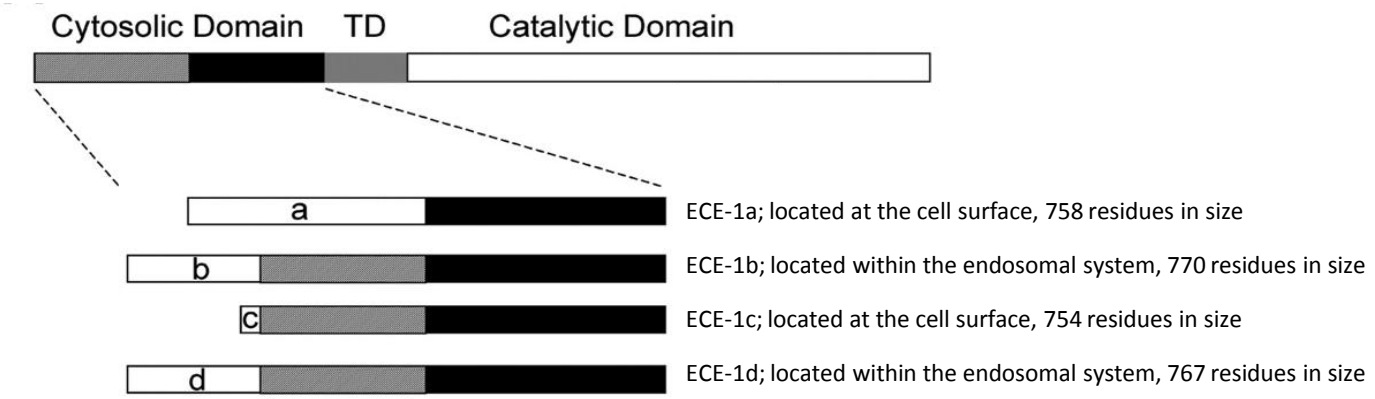


Figure 1.7 The ECE-1 isoforms: A schematic representation of ECE-1 and its four isoforms; -1a. -1b, -1c and -1d. All four isoforms are derived from a single gene, through the use of alternative promoter regions. The N-terminal cytoplasmic region of each isoform differs and is responsible for the targeting of each isoform to its correct location within the cell (Figure adapted from Muller *et al*, 2003).

mitogenic peptides have been related to the disruption of normal cellular homeostasis and has been suggested to contribute to tumour progression by triggering peptide mediated cell proliferation and neoplastic transformation (Papandreou *et al*, 1998). NEP is important in regulating signal transduction processes including cell apoptosis, Akt-mediated survival and cell migration (Sumitomo *et al*, 2005).

1.4.5 Angiotensin II

Angiotensin II (Ang II) has mitogenic and angiogenic effects. The renin-angiotensin system (RAS) is made up of a number of different peptides and their specific receptors. Ang II is the main peptide effector of the RAS. Ang II has two specific GPCRs; AT_1R and AT_2R, in which it can bind to and activate. Ang II exerts most of its pathophysiological effects via AT_1R which is expressed on a number of different cell types. These include cell proliferation, production of growth factors and cytokines and fibrosis, which can lead to vascular thickening and atherosclerosis (Fujiyama *et al*, 2001; Shah and Catt, 2003; Tamarat *et al*, 2002). The AT_2R is mainly expressed during early stages of foetal development, however it is also expressed in the adrenal medulla, ovarian follicles and the uterus during an adult's lifetime (Messerli *et al*, 1996; Touyz *et al*, 2000; Velasquez, 1996). The receptor seems to antagonise the effects of induced proliferation, angiogenesis and inflammatory responses that are activated via the AT_1R (Lung *et al*, 2003; Pupilli *et al*, 1999).

1.4.5.1 The renin-angiotensin system

Ang II is the main precursor peptide of the RAS (Figure 1.8). Other components of the system including the enzymes responsible for the regulation of Ang II are outlined below:

Angiotensin-converting enzyme (ACE) is a zinc-metalloproteinase that is highly conserved in a diverse selection of organisms (Riviere *et al*, 2007; Macours *et al*, 2004). Two forms of the enzyme exist in vertebrates. Somatic ACE is made up of two homologous catalytic domains which both contain the zinc-dependent active site motif HEMGH (Lambert *et al*, 2010). This form of ACE is expressed on the surface of endothelial and epithelial cells in a wide variety of tissues. The other form of the enzyme is germinal ACE. Germinal ACE is made up of a single catalytic domain and is expressed only within the testes. Germinal ACE is known to play an essential role in fertility; its precise function however is not clear (Lambert *et al*, 2010). Both isoforms of the enzyme are type-1 transmembrane glycoproteins which both contain an extracellular amino-terminal ectodomain and a short intracellular cytoplasmic tail. The localisation of ACE to the membrane allows the enzyme to hydrolyse peptides within the extracellular milieu (Lambert *et al*, 2010). ACE has the ability to hydrolyse a number of

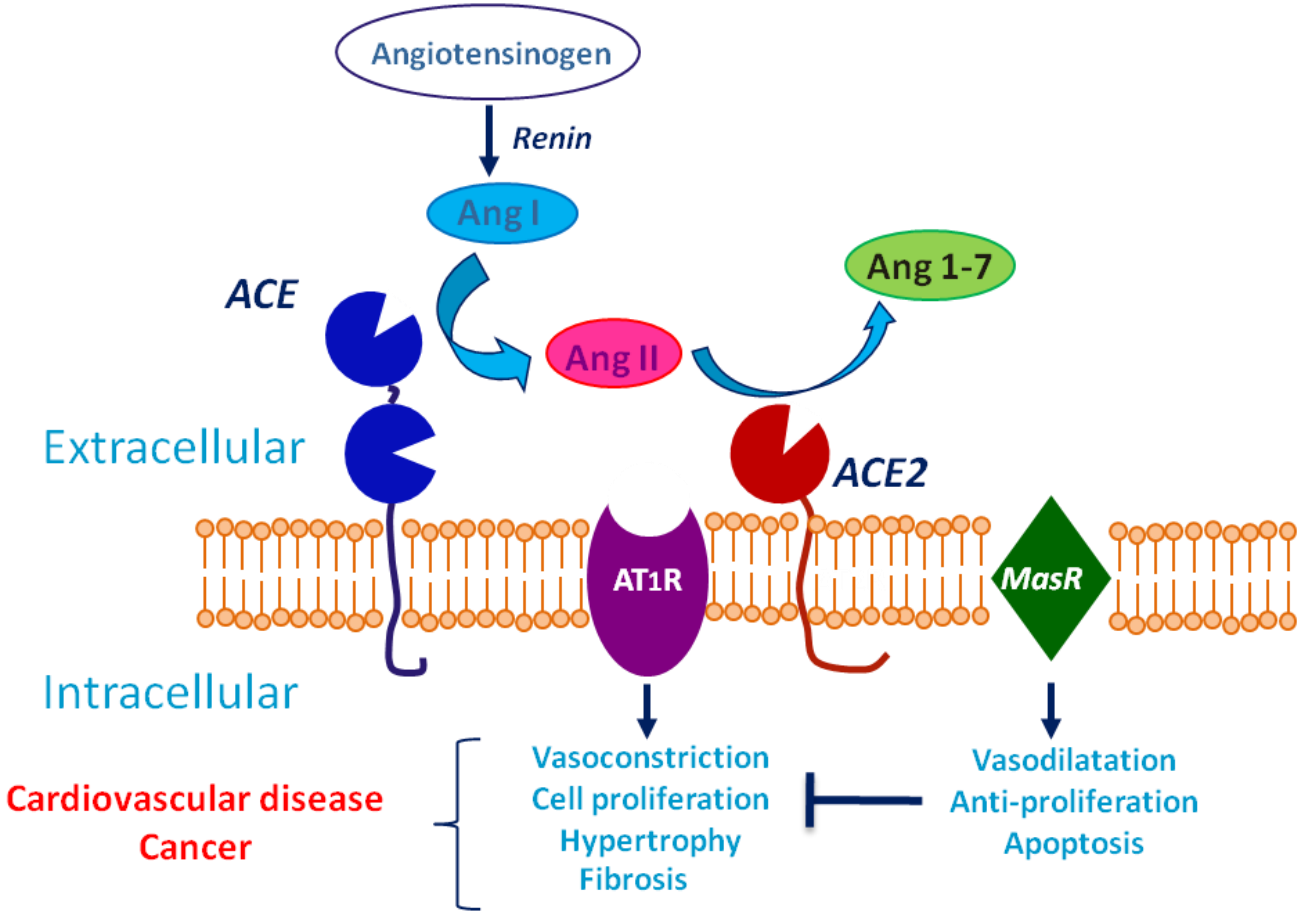


Figure 1.8 The renin-angiotensin system: A schematic diagram to highlight the different components of the RAS. Ang II, produced by the action of ACE, binds to its receptor, AT_1R, to promote cardiovascular disease and cancer. These effects are opposed by Ang 1-7, produced from Ang II by ACE2, acting through its receptor, MasR.

peptides including Ang I, substance P, luteinizing hormone-releasing hormone and BK (Skidgel *et al*, 1984; Skidgel and Erdos, 1985; Rieger *et al*,. 1993). The enzyme can do this by acting as a peptidyl dipeptidase or an endopeptidase. Both domains have different affinities for different substrates and inhibitors (Georgiadis *et al*, 2003).

Angiotensin-converting enzyme 2 (ACE2) is also a zinc-metalloproteinase. It shares 42% sequence homology with ACE. This sequence identity is found within the catalytic domain of both enzymes. ACE2 consists of only one catalytic site and it cleaves its substrates at a specific, single C-terminal residue. ACE2 and ACE have different and distinct substrates. ACE2 and ACE can both cleave Ang I. ACE2 has a lower affinity for the peptide in comparison to ACE and therefore the cleavage event between ACE2 and Ang I is less likely to occur (Rice *et al*, 2004). ACE2 can however cleave Ang II to Ang 1-7 suggesting that the enzyme may act in an opposing way to that in which ACE acts. The enzyme is found throughout many tissues within the body (Gembardt *et al*, 2005) however it is only expressed at high levels in the heart, kidneys and testes (Tipnis *et al*, 2000). ACE2 has a different specificity and physiological role to ACE. Its activity is not altered by the presence of ACE inhibitors. ACE2 is responsible for the production of Ang 1-9 from Ang I. Ang 1-9 is then converted into Ang 1-7 by ACE. ACE2 also has the ability to directly cleave Ang II in order to produce Ang 1-7 (Donoghue *et al*, 2000).

Angiotensin 1-7 (Ang 1-7) is an endogenous, seven amino acid hormone that has both anti proliferative and vasodilator properties (Ferrario *et al*, 1997; Tallent *et al*, 1999; Santos *et al*, 2000). Ang 1-7 can be produced by the enzymatic breakdown of Ang I by a number of endopeptidases including NEP, prolyl oligopeptidase or thimet oligiopeptidase (Welches *et al*, 1991; Yamamoto *et al*, 1992; Chappel *et al*, 1995; Welches *et al*, 1993). This cleavage event takes place at the Pro-7-Phe-8 bond (Welches *et al*, 1993) and it is considered that the event is actually predominantly catalyzed by the ACE2 enzyme. Ang 1-7 can also be produced from Ang II by carboxypeptidases that cleave the mitogenic peptide at its C-terminus. The peptide can also be targeted by the ACE enzyme; however in this case Ang 1-7 is metabolized and degraded to Ang 1-5 (Chappell *et al*, 1998).

The heptapeptide has antiproliferative, diuretic, natriuretic and vasodilatory characteristics (Strawn *et al*, 1999; Ferrario *et al*, 2002; Ren *et al*, 2002; Ferrario, 2003). It can reduce blood pressure and prevent cardiac pathophysiology and attenuation of renal abnormalities that are caused by hypertension (Santos *et al*, 2004; Averil *et al*, 2003; Benter *et al*, 1995; Moriguchi *et al*, 1995; Stegbauer *et al*, 2004). In some instances Ang 1-7 can antagonistic the effects triggered and activated by the binding of Ang II to its receptor, AT_1R (Herath *et al*, 2007).

1.4.5.2 The role of the ET-axis and RAS in cancer progression

Both the ET-axis and RAS have been implicated in cancer progression. The expression of the components within the systems varies in malignant tissue in comparison to normal tissue. The expression of the AT_1R is increased in a number of malignancies. Components of the RAS system have also been linked with tumour grading however no clear correlation has been observed and it very much depends on the tumour type as to how the components vary in malignant tissue to that of normal tissue (Louis *et al*, 2007; Sitzmann *et al*, 1994). In breast cancer over expression of the AT_1R is associated with breast hyperplasia, however the expression levels begin to decrease as the tissue becomes invasive (De Paepe *et al*, 2002). This is not the case in ovarian cancer where an increase in AT_1R expression directly correlates with increased invasiveness (Suganuma *et al*, 2005). Therefore increased invasiveness of malignant tissue may not solely be dictated by an increase in the AT_1R on its own but may require changes in other components of the RAS too (Ager *et al*, 2008).

It is thought that the mitogenic peptides, ET-1 and Ang II, the main precursor peptides of both systems, may play a role in cancer progression by modulating a number of cellular processes (Bagnato and Catt, 1998; Battistin *et al*, 1993; Pirtskhalaishrili and Nelson, 2000).

1.4.5.2.1 Cell proliferation

ET-1 can stimulate cell proliferation via the activation of its specific GPCRs, ET_AR and ET_BR which results in the stimulation of a number of kinases including PKC, IGF-1 and MAPK (Bagnato and Catt, 1998; Battistini *et al*, 1993; Pirtskhalaishrili and Nelson, 2000). The activation of these kinases causes rapid induction and activation of a number of early response genes including c-FOS, c-MYC and c-JUN which can affect the processes of cellular proliferation and apoptosis (Bagnato and Catt, 1998). The transactivation of the EGFR by ET-1 leads to the phosphorylation of the receptor and the phosphorylation of SHC, a signal transduction mediator (Vacca *et al*, 2000). SHC forms a complex with another signal transduction mediator, GRB2. Both SHC and GRB2 can activate p38 and ERK which are both responsible for triggering the change in cell proliferation caused by ET-1 (Vacca *et al*, 2000).

1.4.5.2.2 Cell apoptosis

ET-1 can promote tumour formation and growth by allowing cells to avoid apoptosis. In colon cancer ET-1 acts as a survival factor and inhibits apoptosis that is triggered by Fas ligand binding (Eberl *et al*, 2000; Nelson *et al*, 1999). In ovarian and prostate cancer ET-1 can activate anti-apoptotic signalling pathways including the BCL-2-dependent and PI3-K mediated AKT pathways which result in the activation of BCL-2-associated death promoter (BAD) and Caspase-9 which are both responsible for inhibiting apoptosis and promoting cell growth and survival (Vacca *et al*, 2000; Sumitomo *et al*, 2001).

1.4.5.2.3 Angiogenesis

Angiogenesis is described as the formation of new blood vessels. It is an essential process for the growth and metastasis of solid tumours. HNSCCs are highly invasive especially into surrounding bone and muscle and they also exhibit a high degree of neovascularity (Shemirani and Crowe, 2000). ET-1 and Ang II can both promote angiogenesis. The effects triggered by ET-1 vary depending on which specific GPCR receptor it works through, ET_AR or ET_BR. The activation of the ET_AR can induce zymogen secretion and the induction of mRNA transcription and the pro-enzyme activation of MMPs, which both contribute to tissue remodelling and tumour metastasis. The activation of the receptor by ET-1 can also stimulate the uPA system (Salani *et al*, 2000; Spinella *et al*, 2002; Rosanò *et al*, 2001). The activation of the ET_BR promotes VEGF production and an increase in levels of hypoxia inducible factor-1α (HIF-1α) resulting in increased cell proliferation and vascular permeability both of which are processes associated with tumour angiogenesis and growth (Spinella *et al*, 2002). VEGF is 34-50 kDa in size and is a mitogen associated with endothelial cells that plays a significant role in mediating angiogenesis during both non-pathological and pathological events (Moriyama *et al*, 1997). VEGF is over expressed in a number of cancers (Barr *et al*, 2008; Holtz *et al*, 2008; Kamat *et al*, 2007). Over expression of VEGF and its receptor has been observed in malignant HNSCC therefore suggesting that angiogenesis plays a role in HNSCC development (Shemirani and Crowe, 2000). The VEGF protein and mRNA correlate with malignancy progression (Denhart *et al*, 1997). Ang II and the activation of the ET_BR by ET-1 can increase the production and expression of VEGF by increasing activation of HIF-1 which directly regulates the transcription of VEGF (Page *et al*, 2002).

Ang II can stimulate the expression of a number of growth factors and pro-angiogenic agents that contribute to increased angiogenesis (Folkman, 1975). As mentioned before Ang II can trigger an increase in expression of VEGF, similar to ET-1 (Pupilli *et al*, 1999), an increase in PDGF (Fujita *et al*, 2002), angiopoeitin 2 (Yasumatsu *et al*, 2004) and BFGF (Wysocki *et al*, 2006). The increase in VEGF and endothelial nitric oxide synthase levels also by Ang II can trigger a promotion in revascularization of damaged blood vessels. The increase in expression of these factors is achieved by Ang II through the activation of the AT_1R (Tamarat *et al*, 2002).

There is evidence to support the theory that mitogenic peptides including Ang II and ET-1 may lead to vasoactivity which increases the blood flow to the tumour resulting in an increase in the efficacy of radiotherapy and also improve the delivery of chemotherapeutic drugs (Bell *et al*, 1996; Blackman *et al*, 2003).

Both systems have been targeted for the proliferative activities. The angiogenic effects caused by Ang II can be blocked by the use of ACE inhibitors however side effects of using this drug treatment include pro-angiogenic effects in a number of vascular injuries. This is thought to be due to the increase in BK levels as a result of the inhibition of the ACE enzyme that is responsible for the degradation of the mitogenic peptide (Madeddu *et al*, 2007). It has been suggested that it may therefore be more advantageous to block the receptor in which Ang II binds to, the AT_1R in order to inhibit its proliferative effects (Ager *et al*, 2008). Inhibitors, including perindoprilat, to the ACE enzyme can dramatically reduce the expression levels of VEGF by inhibiting the activity of the VEGF promoter (Yasumatsu *et al*, 2004). The VEGF promoter contains several binding sites. The inhibition of the enzyme can also reduce Ang II levels and lower stromal vascularisation (Yasumatsu *et al*, 2004).

1.4.5.2.4 Migration and invasion

ET-1 is over expressed in both primary and metastatic ovarian carcinomas in comparison to normal ovarian tissues. The activation of the ET_AR by ET can result in the phosphorylation and activation of MAPKs which stimulate the phosphorylation of p125FAK and paxillin. The phosphorylation of these two molecules results in the activation of signals which are thought to be influence migration and invasion of cancerous cells (Vacca *et al*, 2000; Bagnato *et al*, 1997).

1.4.5.2.5 EMT

It ovarian cancer ET-1 acts as a growth factor and is responsible for the activation of signalling pathways that control EMT and promote tumour progression (Rosanò *et al*, 2005). The addition of ET-1 results in the production of an ovarian cancer cell that is spindle like and holds a mesenchymal phenotype (is becoming more migratory and has a fibroblast like shape) instead of a normal polarized phenotype which includes the loss of E-cadherin, β-catenin and ZO-1 and the addition of the mesenchymal markers vimentin and N-cadherin (Rosanò *et al*, 2005).

Fibrosis diseases are characterised by increased ECM formation and the differentiation of myofibroblasts. Fibrosis diseases include liver cirrhosis, pulmonary fibrosis and cardiovascular disease and it is common practice for inhibitors that target components of the RAS system to be used in their treatment. Activated hepatic stellate cells can secrete Ang II. The secretion of Ang II can lead to the promotion of fibrosis, increased myofibroblast proliferation and differentiation and increased collagen synthesis (Bataller *et al*, 2005). α-SMA is also increased and E-cadherin reduced, both of which regulate EMT (Chen *et al*, 1999). Ang II also activates NF-kB which can in turn lead to the increased expression of intracellular adhesion molecules and inflammatory cytokines including TNF-α, IL-6 and TGF-β (Wolf *et al*, 2002; Ozawa *et al*,

2007; Ruiz-Ortega *et al*, 2006). These processes can all promote EMT and therefore contribute to increased invasiveness of the tumour. In response to EMT VEGF expression can be increased as can other growth and angiogenic factors (Bates *et al*, 2007).

1.4.5.2.6 Metastasis

The treatment of ovarian cells with ET-1 increases the production of uPA, the uPA receptor and PAIs at a transcriptional level and an increase in the levels of secretion and activation of MMP-2 and MMP-9 all through the activation of the ET_AR (Rosanò *et al*, 2001). These factors have been linked to an increase in the progression and metastatic ability of ovarian cancerous cells (Rosanò *et al*, 2001).

1.4.5.3 The ET-axis and RAS in head and neck cancer progression

Few studies have investigated the roles that the ET-axis and the RAS play in head and neck cancer progression. Awano *et al* (2006) identified that higher levels of ET-1 were produced in oral squamous cell carcinoma (OSCC) cells compared to normal human epidermal keratinocytes (NHEK) cells and suggested that the peptide may be linked to the development of tumours within the oral cavity via the activation of the ET_AR and/or ET_BR. Higher expression levels of ECE-1, the enzyme responsible for the activation of ET-1, was also observed in OSCC cells compared to normal cells, which may explain the increase in ET-1 levels observed. The activity of ECE-1 could be inhibited by both a specific inhibitor and siRNA to the enzyme. It was shown that an inhibitor specific to ECE-1 inhibited proliferation of OSCC cells as did siRNA targeting of ECE-1 (Awano *et al*, 2006).

The overexpression of ET-1 in the saliva of patients suffering with OSCC has also been observed (Pickering *et al*, 2007). The definite role played by ET-1 in OSCC is not fully understood however Pickering *et al* (2007) suggest that like in other malignancies ET-1 may contribute to the invasive and metastatic properties that OSCC exhibit. ET-1 is also overexpressed in a number of malignancies that have a high incidence of bone metastasis and it has been suggested that this property of ET-1 may contribute to the intense pain associated with OSCC (Pickering *et al*, 2007).

Ishibashi *et al* (2003) demonstrated that high levels of ET-1 in patients suffering with oesophageal SCC, correlated with a poor prognosis. The group identified that a high expression level of VEGF correlated with an increased depth of invasion, the presence of a mutation in p53 and the presence of lymph node metastasis (Ishibashi *et al*, 2003). In ovarian cancer it has been demonstrated that ET-1 stimulates the secretion of VEGF and Ishibashi *et al* (2003) have proposed that the mitogenic peptide may cause the same effect in cases of oesophageal SCC.

mRNA transcripts for components of the RAS including renin, the enzyme responsible for producing Ang I, angiotensinogen, the peptide Ang I is hydrolysed from, ACE, AT_1R and AT_2R have been identified in rat gingival tissue suggesting that the production of Ang II and other vasoactive peptides within this tissue is possible (Santos *et al*, 2009). The presence of all the essential components shows that a local RAS is capable of generating Ang II in rat gingival tissue independently of the systemic RAS (Campbell, 1987; Paul *et al*, 2006; Phillips *et al*, 1993).

1.4.6 Hypothesis and aims

The hypothesis of this thesis is that the deregulation of the ET-axis and RAS contribute to the progression of head and neck cancer.

The overall aim of this study is therefore to investigate the molecular mechanisms used by both ET-1 and Ang II to promote head and neck cancer progression. The expression and function of components of both the ET-axis and RAS will be investigated. The role of the tumour microenvironment in head and neck cancer progression will also be examined with particular focus on the involvement of fibroblasts within this environment.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals routinely used were purchased from Sigma-Aldrich (UK), unless otherwise stated and were of the highest analytical grade.

2.1.2 Antibodies

The monoclonal antibodies to α-smooth muscle actin (α-SMA), clone 1A4 (1:1,000) and β-actin, clone AC-74 (1:4,000) were purchased from Sigma-Aldrich (UK). The anti-human endothelin converting enzyme-1 (ECE-1) (1:500) antibody was purchased from R&D Systems (UK). The antibody is specific to human ECE-1 and does not cross-react with recombinant human ECE-2 or recombinant human membrane metallo-endopeptidases-like 2 enzymes. Anti-mouse, anti-rabbit and anti-goat IgG horseradish peroxidase-conjugated secondary antibodies (1: 2,000) were all purchased from Amersham Ltd (UK).

2.1.3 Peptides and recombinant proteins

Endothelin-1 (ET-1), bradykinin (BK), epidermal growth factor (EGF), angiotensin 1-7 (Ang 1-7) and angiotensin II (Ang II) were all purchased from Sigma-Aldrich (UK). Transforming growth factor (TGF)-β was purchased from R&D Systems (UK).

2.1.4 Oligonucleotides

The following oligonucleotides and primer sequences were synthesized by Sigma-Aldrich (UK).

Primer Name	Forward sequence	Reverse Sequence	Reference
U6	5’ CTCGCTTCGGCAGCACA 3’	5’ AACGTTCACGAATTTGCGT 3’	Baroukh *et al* (2009)
ET_AR	5’ GCTTCCTGGTTACCACTCATCAA 3’	5’ GTCTGCTGTGGGCAATAGTTG 3’	Rayhman *et al* (2008)
ET_BR	5’ TTCATCCCGTTCAGAAGACA 3’	5’ CCAATGGCAAGCAGAAATAGA 3’	Montgomery and Patterson (2008)
ET-1	5’ CTGCCACCTGGACATCATTTG 3’	5’ TCTCACGGTCTGTTGCCTTTG 3’	Rayhman *et al* (2008)
ECE-1	5’ GGACTTCTTCAGCTACGCCTGT 3’	5’ CTAGTTTCGTTCATACACGCACG 3’	Kindly donated by Dr. L. Lambert (University of Leeds)
NEP	5’ CCTGGAGATTCATAATGGATCTTGT 3’	5’ AAAGGGCCTTGCGGAAAG 3’	Kindly donated by Dr. L. Lambert (University of Leeds)
COX-2	5’ GCTCAGCCATACAGCAAATC 3’	5’ TGTGTTTGGAGTGGGTTTCA 3’	Wakimoto *et al* (2008)
AT_1R	5’ ACCTGGCTATTGTTCACCCAA 3’	5’ ACAAGCATTGTGCGTCGAAG 3’	Dubois *et al* (2010)
AT_2R	5’ GTTCCCCTTGTTTGGTGTAT 3’	5’ CATCTTCAGGACTTGGTCAC 3’	Okamura *et al* (1999)
MasR	5’ TTCCGGATGAGAAGAAATCC 3’	5’ ATGGCCAGAAGAAAGCTCAT 3’	Reis *et al* (2011)
ACE	5’ ATGAAGACCTGTTATGGGCATGG 3’	5’ ATTTCGGGTAAAACTGGAGGATGG 3’	Nakamura *et al* (2011)
α-SMA	5’ GAAGAAGAGGACAGCACTG 3’	5’ TCCCATTCCCACCATCAA 3’	Yue *et al* (2008)

Table 2.1 Oligonucleotide sequences

2.1.5 Bacterial strains

In order to multiply the quantity of expression vectors the DH5α *E.coli* strain was used (New England Biolabs, UK).

2.1.6 Plasmids

The mammalian expression vector containing cDNA encoding human ECE-1c (complete coding sequence of human ECE-1c inserted by blunt-ended ligation into pcDNA3) was kindly donated by Dr. A. Whyteside (University of Leeds) and the mammalian expression vector containing cDNA encoding human ACE (complete coding sequence of human somatic ACE inserted by blunt-ended ligation into pIRES-neo) was kindly provided by Dr. E. Parkin (University of Lancaster).

2.1.7 Cells

The cell lines used in this study are listed below:

Cell Line	Derivation	Source	Reference
Cal27	Tongue, OSCC	Dr. C. Murdoch	Gioanni *et al* (1988)
H357	Tongue, OSCC	Dr. S. Whawell	Prime *et al* (1990)
SCC4	Tongue, OSCC	Dr. C. Murdoch	Rheinwald and Beckett (1980)
FaDu	Pharyngeal, OSCC	Dr. C. Murdoch	Rangan (1972)
B16	Tongue, OSCC	Dr. K. Hunter	Burns *et al* (1993)
D19	Lateral tongue, Dysplasia	Dr. K. Hunter	McGregor *et al* (2002)
D20	Lateral tongue, Dysplasia	Dr. K. Hunter	McGregor *et al* (2002)
B22	Metastasis	Dr. K. Hunter	Burns *et al* (1993)
Normal oral keratinocytes (NOKs)		Collected with South Sheffield ethics committee approval, as described in Hearnden *et al* (2009)	
Normal oral fibroblasts (NOFs)		Collected with South Sheffield ethics committee approval, as described in Hearnden *et al* (2009)	

Table 2.2 Head and neck primary cells and cell lines utilised

2.1.8 Cell culture reagents

Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium (RPMI-1460), Ham's F12 nutrient mix, foetal bovine serum (FBS), Dulbecco's phosphate buffered saline (PBS), L-glutamine, adenine, transferrin, 3,3',5-triiodo-L-thyronine, hydrocortisone and trypsin/EDTA were all purchased from Bio Whitaker (UK). The transfection reagents Oligofectamine and FuGENE 6 were bought from Invitrogen (UK) and Promega (UK), respectively. Transwell inserts and companion plates were purchased from Corning (UK). Matrigel was purchased from Becton Dickinson (UK).

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Routine maintenance of cell culture

The HNSCC-derived cell lines Cal27, H357 and SCC4 and human primary NOFs (used within passage 3-10) were routinely cultured in DMEM supplemented with 2 mM L-glutamine and 10% (v/v) FBS. The FaDu cell line was routinely grown in RMPI-1460 supplemented with 2 mM L-glutamine and 10% (v/v) FBS. Human primary NOKs (used up to passage 3) and D19, D20, B16 and B22 cell lines were all routinely cultured in Green's media consisting of a 3:1 ratio of DMEM and Ham's F12 supplemented with 2mM L-glutamine, $1.8x10^{-4}$ M adenine, 5 µg/mL transferrin, $2x10^{-7}$ M 3,3',5-triiodo-L-thyronine, 4 µg/mL hydrocortisone, 10 ng/mL EGF and 10% (v/v) FBS. All cells were grown in antibiotic-free media at 37°C and 5 % (v/v) CO_2. Following two washes with PBS, cells were routinely passaged using trypsin/EDTA digestion. For cell line references please see Table 2.2.

2.2.1.2 Cryogenic preservation and recovery

Cells at ~90% confluency were washed twice in PBS before being harvested using trypsin/EDTA digestion. Cells were resuspended in normal growth media containing 50% (v/v) FBS and 10% (v/v) DMSO. $1x10^6$ cells were divided into 1 ml aliquots and added to cryotubes. The tubes were placed at -80°C in a cryo-cooler and left overnight. For long-term storage the cells were placed into liquid nitrogen.

2.2.2 Cell treatments

2.2.2.1 Preparation of conditioned media

NOFs were seeded at 300,000 cells/well in a 6-well plate in DMEM containing 10% (v/v) FBS and were left to adhere overnight at 37°C and 5% (v/v) CO_2. The following day NOFs were serum starved for 24 h in DMEM.

Cells were treated with ET-1 (0-100 nM (Figure 4.1B)), BK (1 μM (Clementi *et al*, 1999)), Ang 1-7 (100 nM (Zhang *et al*, 2010)) and/or Ang II (0-1000 nM (Figure 4.3A)) or TGF-β (2-20 ng/ml (Kellermann *et al*, 2008)) and incubated for 4-48 h at 37°C and 5% (v/v) CO_2. Where indicated, NOFs were pre-treated with an ET_AR antagonist, an ET_BR antagonist (BQ-123 and BQ-788, respectively, both 1 μM (Spinella *et al*, 2002); Sigma-Aldrich, UK) individually or in combination, an MMP/ADAMs inhibitor (GM6001, 10 μM (Mei *et al*, 2001); Chemicon), an AT_1R antagonist (Telmisartan, 1 μM (Storka *et al*, 2008); Sigma-Aldrich, UK), a mas receptor (MasR) antagonist (A-779, 1 μM (Zhang *et al*, 2010); Bioquote) or an ECE-1 inhibitor (SM-19712, 10 μM (Roosterman *et al*, 2008); Sigma-Aldrich, UK), for 30 min before addition of mitogenic peptides. Media was collected from the NOFs after incubation, filtered and added either directly to SCC4 cells (for COX-2 expression analysis) or to the bottom well of a Transwell chamber (for migration and invasion assays). Where appropriate, the conditioned media was incubated for 30 min at 37°C with rotation following addition of neutralising antibodies to HB-EGF, TGF-α and amphiregulin (10 μl/ml, all R&D Systems, UK) or mouse IgG (Dako) before addition to the Transwell.

2.2.2.2 Treatment of HNSCC cell lines

SCC4 or H357 cells were serum starved 24 h prior to experimentation in DMEM. Cells were treated with ET-1 (10 nM), BK (1 μM), EGF (48.4 μM (Fowler *et al*, 1995)), Ang 1-7 (100 nM) and/or Ang II (100 nM) or TGF-β (2-20 ng/ml). Where indicated SCC4 or H357 cells were pre-treated with an AT_1R antagonist (Telmisartan, 1 μM), an AT_2R antagonist (PD123319, 500 nM (Mlinar *et al*, 1995); Sigma-Aldrich, UK), a MasR inhibitor (A-779, 1 μM), an EGFR antagonist (Tyrphostin, AG 1478, 125 nM (Pierce *et al*, 2000); Sigma-Aldrich, UK), an ECE-1 inhibitor (SM-19712, 10 μM), non-specific COX inhibitor (Ibuprofen, 100 μM (Ouellet *et al*, 2001); Sigma-Aldrich, UK) or specific COX-2 inhibitor (Celecoxib, 50 μM (Niederberger *et al*, 2001); Sigma-Aldrich, UK) for 30 min at 37°C and 5% (v/v) CO_2 before the experimental use of the cells.

The inhibitors and antagonists used in this study are listed in Table 2.3.

Name	Chemical name	K_d	IC_{50}	Other information	Reference
EGFR antagonist	**AG1478**		3 nM	Is a specific inhibitor of EGF-receptor tyrosine kinase activity, can also abolish EGFR autophosphorylation and Src family kinase.	Osherov and Levitzki, 2002
AT_1R antagonist	**Telmisartan**	1.7 (± 1.2) nM		Telmisartan dissociates very slowly from AT_1R, When administered intravenously in rats, telmisartan is 10-fold more potent than irbesartan and comparable to candesartan, two other AT_1R antagonists.	Maillard *et al*, 2002
AT_2R antagonist	**PD123319**	0.09 nM	30 nM	Completely inhibits AT_2R activity in a monophasic manner.	Servant *et al*, 1993
MasR inhibitor	**A-779**		10 µM	A-779 does not change the dipsogenic, pressor, or myotropic effects of Ang II an does not affect the antidiuretic effect of vasopressin or the contractile effects of angiotensin III, bradykinin, or substance P on the rat ileum therefore highlighting it is a potent and selective antagonist for Ang 1-7.	Santos *et al*, 1994
ECE-1 inhibitor	**SM-19712**		42 nM	SM-19712 inhibited ECE activity but had no effect on other metalloproteases such as NEP and ACE, showing that it has a high specificity for ECE.	Umekawa *et al*, 2000
COX inhibitor	**Ibuprofen**		57 µM	Has a time independent mechanism and is selective towards COX-1.	Noreen *et al*, 1997
COX-2 inhibitor	**Celecoxib**		0.04 µM	Has an IC_{50} of 15 µM for COX-1. Can demonstrate potent, anti-inflammatory activity, has potency equivalent to standard NSAIDs and showed no acute GI toxicity, has good bioavailability, is well distributed and has an excellent safety profile.	Penning *et al*, 1997
ET_AR antagonist	**BQ-123**		7.3 nM	BQ-123 interacts competitively with ET_AR.	Ihara *et al*, 1992
ET_BR antagonist	**BQ-788**		1.2 nM	BQ-788 interacts competitively with ET_BR and it does not significantly inhibit the binding of Ang II or calcitonin-gene related peptide.	Ishikawa *et al*, 1994
MMP/ADAMs inhibitor	**GM6001**	0.18-26.0 nM	0.104-263.0 nM	Inhibits a wide range of MMPs including MMP-2, MMP-3, MMP-26, MMP-8, MMP-9, MMP-1, MMP-12, MMP-14, MMP-15, MMP-16, ADAM-9, ADAM-10, ADAM-12 and ADAM-17.	www.drugable.com

Table 2.3 Inhibitor and antagonist information

2.2.2.3 COX-2 analysis

SCC4 cells were seeded at 100,000 cells/well in a 12-well plate in DMEM containing 10% (v/v) FBS and were left to adhere overnight at 37°C and 5% (v/v) CO_2. The following day SCC4 cells and NOFs were serum starved for 24 h in DMEM.

NOFs were treated and incubated with ET-1 (10 nM) for 4 h at 37°C and 5% (v/v) CO_2. SCC4 cells were subsequently stimulated with the conditioned NOFs media for a further 4 h before the cells were lysed, total RNA isolated (as described in Section 2.2.3.4) and cDNA analysed by SYBR green qPCR (as described in Section 2.2.3.4).

2.2.3 Molecular analysis

2.2.3.1 Transformation of competent cells

Chemically competent DH5α *E.coli* cells (New England Biolabs, UK) were thawed on ice. Plasmid DNA (1 pg-100 ng) was added to the competent cells. The cells and DNA were flicked 4-5 times in order to mix the solution. The mixture was incubated on ice for 2 min. The bacteria were subjected to heat shock treatment at 42°C for 30 s before being returned to ice for a further 2 min. 950 µl of super optimal broth with catabolite repression (S.O.C) medium (Invitrogen, UK) was added to the transformation mix. 50-100 µl of the transformed mix was added to Luria-Bertani (LB)-agar (Fisher Scientific, UK) plates supplemented with 50 µg/ml ampicillin (Sigma-Aldrich, UK) and incubated overnight at 37-42°C in order to select for successful transformants.

2.2.3.2 Plasmid maxi prep from transformants

Cells harbouring the plasmid were cultured in LB liquid media (Fisher Scientific, UK) supplemented with ampicillin (50 µg/ml). The mixture was incubated overnight at 37°C with agitation. The cells were harvested using centrifugation at 10,000 *g* for 15 min. DNA was isolated from the bacterial cells using the Qiagen Spin Maxiprep kit (UK) according to manufacturer's instructions. DNA from each sample was quantified using a NanoDrop spectrophotometer (Fisher Scientific, UK).

2.2.3.3 Transfection

2.2.3.3.1 Transient transfection of siRNA or miRNA in cells

Cells were seeded in 6-well plates in DMEM containing 10% (v/v) FBS and were left to adhere overnight at 37°C and 5% (v/v) CO_2. The following day cells at approximately 60% confluency were transfected using Oligofectamine reagent (Invitrogen, UK). 10 µl Oligofectamine was added to Opti-MEM medium (Invitrogen, UK) to a final volume of 50 µl. This mixture was

added to siRNA targeted to ADAM17, microRNA (miR)-145, a negative siRNA control or a negative miRNA control (Applied Biosystems) prepared to a final concentration of 50 nM in 50 µl Opti-MEM. The Oligofectamine and siRNA or miRNA mixture was left to incubate at room temperature for 30 min before addition to NOFs. Prior to transfection, NOFs were washed twice in 1 ml volumes of Opti-MEM medium. The transfection mixture was added to the washed NOFs and left for 4 h at 37°C and 5 % (v/v) CO_2. 500 µl of Opti-MEM was added to the cells and they were incubated for 24 h and media and cells collected for further experimentation and/or analysis, as described below.

2.2.3.3.2 Transient transfection of plasmid in cells

Cells were seeded in 6-well plates in DMEM containing 10% (v/v) FBS and were left to adhere overnight at 37°C and 5% (v/v) CO_2. The following day cells at approximately 60% confluency were transfected using FuGENE 6 transfection reagent (Promega, UK). 3 µl FuGENE 6 was added to Opti-MEM medium to a final volume of 50 µl. This mixture was added to DNA which had been prepared to a final concentration of 1 µg in 50 µl in Opti-MEM medium (a 3:1 ratio of FuGENE 6:DNA). The FuGENE 6 and DNA mixture was left to incubate at room temperature for 20 min before its addition to the SCC4 cells or NOFs. Prior to transfection, SCC4 cells or NOFs were washed twice in 1 ml volumes of Opti-MEM medium. The transfection mixture was added to the washed cells and left for 4 h at 37°C and 5% (v/v) CO_2. 500 µl of Opti-MEM was added to the cells and they were incubated for 24 h and media and cells collected for further experimentation and/or analysis, as described below.

2.2.3.4 RNA isolation and quantitative real-time PCR

Total RNA was isolated from cell lines, and primary NOKs and primary NOFs using the RNeasy mini kit (Qiagen, UK) according to the manufacturer's instructions. RNA from each sample was quantified using a NanoDrop spectrophotometer (Fisher Scientific). High Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used for synthesis of cDNA according to the manufacturer's instructions. The following RT-PCR programme was used for total RNA for SYBR green analysis: 25°C for 10 min, 37°C for 120 min, 90°C for 5 min. The following RT-PCR programme was used on RNA for miRNA analysis: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min. A 2720 Thermal Cycler from Applied Biosytems was used to conduct the cDNA synthesis. cDNA was subsequently analysed by SYBR green qPCR or Taqman qPCR using a Step One real-time PCR system thermal cycling block (Applied Biosystems). The Standard Thermal Cycle protocol was used. The cycling parameters were 40 cycles with each cycle being comprised of heating to 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec and 60°C for 1 min.

For primer sequences used for SYBR green quantification please see Table 2.1. Taqman probes for ADAM17, ACE2, miR-145 and β2microglobulin were obtained from Applied Biosystems. All values were normalised to U6, a small nuclear ribonucleoprotein, (SYBR) or β2microglobulin (Taqman) expression levels and the relative expression of each transcript was calculated using the *ΔΔCT* method (Livak and Schmittgen, 2001). Where indicated, amplicons were also analysed by agarose gel electrophoresis (as described in Section 2.2.3.5) and visualised with ethidium bromide under UV transillumination.

2.2.3.5 Agarose gel electrophoresis

Samples were separated by electrophoresis on a 1.5% (w/v) agarose gel in 1 X TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) containing ethidium bromide to a final concentration of 5 µg/ml. Samples were loaded in gel-loading buffer (0.25% (w/v) bromophenol blue, 40% (w/v) sucrose in H_20) and electrophoresed in 1 X TAE at 100 V. Bands were visualised under UV transillumination and HyperLadder I (Bioline, UK) was used as a molecular weight standard.

2.2.4 Cell migration assay

Migration was monitored using Transwell Permeable Supports with a PET track etched membrane containing 8 µm pores (Corning, UK). SCC4 or H357 at 90% confluency were serum starved for 24 h prior to experimentation. Cells were trypsinised and resuspended in serum free DMEM. Cells were pelleted at 200 *g* for 5 min and resuspended in DMEM containing 0.1% (w/v) BSA at $5x10^5$ cells/ml. Cells were either left untreated or were treated with ET-1 (10 nM), BK (1 µM), EGF (48.4 µM), Ang II (100 nM) and/or Ang 1-7 (100 nM) and 200 µl cell suspension was added to the top of the Transwell insert.

The EGFR antagonist (AG 1478, 125 nM), AT_1R antagonist (Telmisartan, 1 µM), AT_2R antagonist (PD123319, 1 µM), MasR antagonist (A-779, 1 µM), ECE-1 inhibitor (SM-19712, 10 µM), non-specific COX inhibitor (Ibuprofen, 100 µM) or specific COX-2 specific inhibitor (Celecoxib, 50 µM) were incubated with the SCC4 cell suspension for 30 min at 37°C and 5% (v/v) CO_2 before the addition of the SCC4 cell suspension to the Transwell insert. 500 µl of DMEM containing conditioned media collected from NOFs pre-treated for 30 min with ET_AR antagonist (BQ-123, 1 µM) and/or ET_BR antagonist (BQ-788, 1 µM), AT_1R antagonist (Telmisartan, 1 µM), AT_2R antagonist (PD123319, 1 µM), MasR antagonist (A-779, 1 µM), MMP/ADAMs inhibitor (GM6001, 10 µM), ECE-1 inhibitor (SM-19712, 10 µM) before the addition of ET-1 (0-100 nM), BK (1 µM), Ang 1-7 (100 nM) and/or Ang II (0-1000 nM) or TGF-β (2-20 ng/ml) for 4-48 h at 37°C and 5% (v/v) CO_2 (as described in individual figure legends) or DMEM containing 10% (v/v) FBS was added to the bottom well of the Transwell chamber to act as an attractant. The

EGFR inhibitor was also added to the media collected from the NOFs before it was added to the bottom of the Transwell chamber.

Migration assays were left for 16 h at 37°C and 5% (v/v) CO_2. After the incubation period, cells were swabbed away from the inside of the migration insert and media was removed by suction from the bottom of the Transwell chamber. Cells adhering to the underside of the insert were fixed for 10 min in 500 µl 100% (v/v) methanol, added to the top and bottom of the chamber. Cells were washed in 500 µl volumes of PBS and were stained with 500 µl volumes of 0.1% (w/v) crystal violet for 20 min. Cells were washed and left in PBS and enumerated by light microscopy at 40x magnification. Three fields of view from each insert were counted and an average taken.

2.2.5 Cell invasion assay

Invasion was monitored using Transwell Permeable Supports (Corning, UK) containing 100 µl of growth factor-depleted Matrigel (BD Biosciences) diluted 1:45 in serum free DMEM (Lang *et al*, 2000). Invasion assays were prepared 24 h prior to experimentation and left overnight at 37°C and 5% (v/v) CO_2 to allow the Matrigel to set.

SCC4 or H357 at 90% confluency were serum starved for 24 h prior to experimentation. Cells were trypsinised and resuspended in serum free DMEM. Cells were pelleted at 200 *g* for 5 min and resuspended in DMEM containing 0.1% (v/w) BSA at $5x10^5$ cells/ml. Cells were either left untreated or were treated with ET-1 (10 nM), Ang II (100 nM) and/or Ang 1-7 (100 nM) and 200 µl cell suspension was added to the top of the Transwell chamber. The Matrigel was not removed from the wells before the cells were added. 500 µl of DMEM containing conditioned media collected from NOFs pre-treated for 30 min with ET_AR antagonist (BQ-123, 1 µM) and/or ET_BR antagonist (BQ-788, 1 µM) or AT_1R antagonist (Telmisartan, 1 µM) before further treatment with ET-1 (10 nM), BK (1 µM), Ang 1-7 (100 nM) and/or Ang II (100 nM) for 4-48 h at 37°C and 5% (v/v) CO_2 (as described in individual figure legends) or DMEM containing 10% (v/v) FBS was added to the bottom well of the Transwell chamber to act as an attractant.

Invasion assays were left for 40 h at 37°C and 5% (v/v) CO_2. After the incubation period, cells and Matrigel were swabbed away from the inside of the invasion insert and media was removed by suction from the bottom of the Transwell chamber. Cells adhering to the underside of the insert were fixed for 10 min in 500 µl 100% (v/v) methanol, added to the top and bottom of the chamber. Cells were washed in 500 µl PBS and were stained with 500 µl 0.1% (w/v) crystal violet for 20 min. Cells were washed and left in PBS and enumerated by

light microscopy at 40x magnification. Three fields of view from each insert were counted and an average taken.

2.2.6 Cell proliferation assay

Proliferation assays were carried out in 96-well plates. NOFs were trypsinised, resuspended in DMEM containing 10% (v/v) FBS, counted and seeded at 2,000 cells/well. Cells were left to adhere overnight at 37°C and 5% (v/v) CO_2.

The following day media was removed and wells were washed in 100 µl PBS. Cells were incubated with an ET_AR antagonist (BQ-123, 1 µM) and/or an ET_BR antagonist (BQ-788, 1 µM) or a water control in 500 µl of serum free DMEM for 30 min before the addition of ET-1 (10 nM). At each time point 20 µl of MTS reagent (Promega, UK) was added to each well and the plate was incubated for 1 h at 37°C and 5% (v/v) CO_2. A fluorescence reading was recorded at 492 nm using a fluorescence spectrophotometer (Tecan).

2.2.7 Gel contraction assay

Fibroblast:collagen lattices were made in 24-well plates. NOFs (250,000/well) were resuspended in DMEM containing 10% (v/v) FBS and were mixed with rat-tail collagen (7.5 mg/ml) and 10X DMEM. The pH of the mixture was adjusted to pH 7 using 0.1 M NaOH and to a final volume of 300 µl. 300 µl of the fibroblast:collagen mixture was added to each well and allowed to incubate for 45 min. 500 µl of DMEM containing 10% (v/v) FBS was then added carefully to each well and the lattices were allowed to incubate at 37°C and 5% (v/v) CO_2 for 4 h. After this time period the media from each well was carefully removed and replaced with 500 µl serum free media. The lattices were left to incubate at 37°C and 5% (v/v) CO_2 overnight.

The following day the serum free DMEM media was removed from each well and a scalpel was used to carefully detach the collagen lattice from the sides of the well. Each lattice was immediately incubated with an ET_AR antagonist (BQ-123, 1 µM), an ET_BR antagonist (BQ-788, 1 µM) or a water control in 500 µl of serum free DMEM for 30 min before the addition of either ET-1 (10 nM), Ang II (100 nM), DMEM containing 10% (v/v) FBS, thrombin (0.5 units/ml; Sigma-Aldrich, UK).

Each lattice was photographed 30 min after treatment and the distance contracted by each gel was measured.

2.2.8 Scratch assay

Scratch assays were carried out in 12-well plates. NOFs were seeded at 100,000 cells/well in DMEM containing 10% (v/v) FBS and were left to adhere overnight at 37°C and 5% (v/v) CO_2. The following day the media was removed and replaced with serum free DMEM and the cells were again left to incubate overnight.

A horizontal 'scratch' was made across each well using the sharp end of a 200 µl pipette tip. The media was removed and the cells were washed twice in 1ml volumes of PBS. Cells were incubated with receptor antagonists an ET_AR antagonist (BQ-123, 1 µM) and/or an ET_BR antagonist (BQ-788, 1 µM) or a water control in 500 µl of serum free DMEM for 30 min. The cells were then treated with either ET-1 (10 nM), Ang 1-7 (100 nM) and/or Ang II (100 nM), DMEM containing 10% (v/v) FBS, TGF-β (2-20 ng/ml). Mitomycin C (Sigma-Aldrich, UK) at 1 µg/ml was also added to each well to prevent cellular proliferation. Each well was photographed at two points along the scratch at 0 h and 24 h. The cells were allowed to incubate at 37°C and 5% (v/v) CO_2 between time points. The distance between each edge of the scratch was measured in order to determine the distance migrated by the NOFs.

2.2.9 Protein analysis

2.2.9.1 Preparation of cell lysates

Cells were washed twice in PBS before being trypsinised and resuspended in DMEM containing 10% (v/v) FBS. Cells were pelleted in a centrifuge at 2000 *g* for 5 min. The supernatant was discarded and the pellet was re-suspended in PBS and repelleted by centrifugation. The supernatant was again discarded. Pellets were resuspended in varying volumes of lysis buffer containing RIPA buffer (10X) (Cell Signaling Technology, Inc) containing Complete Mini Protease Inhibitor Cocktail (Roche (Fisher Scientific, UK); used according to manufacturer's instructions) and Benzonase (Sigma-Aldrich, UK; used according to manufacturer's instructions). Samples were left on ice for 30 min and then centrifuged at 13000 *g* for 2 min before the supernatant was removed and collected and the pellet was discarded.

2.2.9.2 Protein concentration assay

Protein concentration was determined BCA Protein Assay Kit (Fisher Scientific; used according to manufacturer's instructions) with bovine serum albumin (BSA; 1 mg/ml) acting as a standard (Laemmli, 1970).

2.2.10 SDS-PAGE and western blotting

2.2.10.1 Preparation of samples

Total protein extracts (50 µg) were run in each well. LDS sample buffer (4X, Invitrogen, UK) and reducing agent (10X, Invitrogen, UK) were both added to each sample before it was placed on a heating block at 100°C for 10 min. The sample was pulsed in a microcentrifuge.

2.2.10.2 Gel electrophoresis

Proteins were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 3-8% (v/v) polyacrylamide Tris-Acetate gels (Invitrogen, UK) or 4-12% (v/v) polyacrylamide Bis-Tris gels (Invitrogen, UK). Tris-Acetate gels were washed in Tris-Acetate SDS Running Buffer (20X, Invitrogen, UK) and Bis-Tris gels were washed in MES SDS Running Buffer (20X, Invitrogen, UK). Both buffers contained NuPAGE antioxidant (Invitrogen, UK; used according to manufacturer's instructions). Gels were placed in an Invitrogen Mini Cell and protein samples were loaded to the correct well. The centre chamber of the cell and the outside of the cell were filled with Tris-Acetate SDS Running Buffer 20X or MES SDS Running Buffer containing NuPAGE antioxidant. Tris-Acetate gels were run for 1 h at 150 V and Bis-Tris gels were run for 35 min at 200 V.

2.2.10.3 Electrotransfer:

Sponge pads, a nitrocellulose membrane and filter paper were soaked in NuPAGE Transfer Buffer (Invitrogen, UK) containing 10% (v/v) methanol per gel beforehand. For the electrotransfer of each gel, sponge pads were placed into the cassette followed by a piece of filter paper, Whatman Optitran reinforced nitrocellulose membrane (Sigma-Aldrich, UK), the gel and another piece of filter paper followed by more pads. Each gel was transferred for 1 h at 30 V.

2.2.10.4 Immunodetection

Nitrocellulose membranes were non-specifically blocked using tris-buffered saline (10 mM Tris-HCl, pH 7.4) containing 0.5% (v/v) Tween 20 (TBS-T) with 5% (w/v) milk powder (Marvel, UK) and 3% (w/v) BSA (Sigma-Aldrich, UK) for 1 h at room temperature on a rocking platform. Membranes were incubated with the necessary primary antibody (β-actin (1:4,000), α-SMA (1:1,000), ECE-1 (1:500)) for 1-3 h at room temperature or overnight at 4°C on a rocking platform. Nitrocellulose membranes were washed three times in TBS-T at 10 min intervals. The membrane was incubated with anti-mouse, anti-rabbit or anti-goat IgG horseradish peroxidase secondary antibody at 1:2,000 for 1 h at room temperature on a rotating surface. The nitrocellulose membrane was then washed three times in TBS-T at 15 min intervals.

2.2.10.5 Development of western blots

Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Amersham Ltd., Amersham, UK). Reagents A and B, in a 1:1 ratio, were mixed together and the nitrocellulose membrane submerged in the solution for 1 min at room temperature. Each membrane was covered in Saran wrap and developed using CL-XPosure film (Fisher Scientific) and Compact X4 automatic processor (Xograph Imaging Systems).

2.2.10.6 Stripping and reprobing of membranes

Membranes were routinely stripped and reprobed with β-actin in order to determine loading efficiency. Membranes were incubated with 50 ml stripping buffer (62.5 mM Tris pH 6.7, 2% SDS and 100 mM 2-βmecaptoethanol) at 50°C for 30 min. Membranes were washed four times in large volumes of TBS-T at 15 min intervals and subjected to immunoblotting as described in Section 1.2.10.3.

2.2.11 Statistical analyses

Data are expressed as the mean ± SEM. Each experiment was carried out in technical repeat and where appropriate three different patient samples were used. Normal distribution of the data was assessed using the Sharpio-Wilk test; statistical analyses were made between two groups using a parametric Student *t*-test. A value of $p<0.05$ was considered significant.

Chapter 3: The effect of mitogenic peptides on autocrine signalling in head and neck cancer progression

3.1 Introduction

Endothelin-1 (ET-1), bradykinin (BK) and angiotensin II (Ang II) are all examples of small regulatory peptides that have been implicated in the progression of a number of malignancies. Many of these peptides originate from precursor molecules that have undergone posttranslational modifications involving several enzymatic events, resulting in the production of a mature peptide (Siegfried *et al*, 1992). They often act as ligands for specific G-protein coupled receptors (GPCRs) and can signal via an autocrine mechanism in which they act on the same cell that they are secreted from. The activation of specific GPCRs by mitogenic peptides can trigger many intracellular signalling pathways. Alteration of these signalling pathways can lead to deregulated cell behaviour and function.

ET-1 is a mitogenic peptide which acts primarily as a potent endogenous vasoconstrictor (Yanagisawa *et al*, 1988) but also has various other physiological roles including the maintenance of basal vascular tone, central respiratory regulation and maintaining renal homeostasis. There are three members of the ET family: ET-1, ET-2 and ET-3. Each peptide is made up of 21 amino acids (Inoue *et al*, 1989). ET-1 is the most abundant ET and is the product of the cleavage of pre-pro ET-1 to big ET-1 by a furin-like endopeptidase, and then the cleavage of big ET-1 to ET-1 by endothelin converting enzyme-1 (ECE-1); a membrane bound metalloproteinase (Rubanyi and Polokoff, 1994). This second proteolytic cleavage by ECE-1 occurs between the Trp-21-Val/Ile-22 bond. It is an essential step in the activation of ET-1 because its precursors have no biological activity (Rubanyi and Polokoff, 1994). The actions of ET-1 are mediated by the binding of the peptide to two GPCRs; ET_AR and ET_BR (Arai *et al*, 1990; Sakurai *et al*, 1992).

ET-1 is produced at elevated levels in cancer cells (Levin, 1995) and various lines of evidence suggest that the peptide can play an important role in cancer progression (Nelson and Carducci, 2000). The binding of ET-1 to ET_AR and/or ET_BR in cancer cells by ET-1 results in the activation of downstream kinases and the stimulation of cellular signalling and proliferation. Kinases including protein kinase C (PKC) and mitogen-activated protein kinase (MAPK), factors including epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 (IGF-1) and early response genes including c-myc, c-fos and c-jun can all be activated by ET-1 stimulation (Battistini *et al*, 1993; Bagnato *et al*, 1997; Pirtskhalaishvili and Nelson, 2000). GPCR ligands can transactivate the EGFR and the ability of gastrin-releasing peptide (GRP) receptor to activate the EGFR has been implicated in the modulation of head and neck squamous cell carcinoma (HNSCC) growth and invasion (Lui *et al*, 2003). The mitogenic peptide is also known to stimulate angiogenesis by directly acting as a chemoattractant for endothelial cells and vascular smooth muscle cells (Salani *et al*, 2000) and by indirectly recruiting vascular

endothelial growth factor (VEGF) (Spinella *et al*, 2002). ET-1 can also promote tumour growth by suppressing the apoptosis of cancer cells (Eberl *et al*, 2000). ET-1 is also able to promote chemotaxis of prostate and ovarian cancer cells (Nelson *et al*, 1996; Bagnato *et al*, 1995). Elevated levels of the peptide have been detected in oral specimens and in the saliva of patients diagnosed with oral cancer (Pickering *et al*, 2007). Pickering *et al* (2007) also identified a link between ET-1 and bone metastasis in HNSCC. Awano *et al* (2006) have also observed that ET-1 can promote an increase in proliferation of HNSCC cell lines and elevated levels of ET-1 have been associated with a poor prognosis (Ishibashi *et al*, 2003). ET-1, ECE-1; the enzyme responsible for its activation and the receptors it exerts its effects through are collectively described as the ET-axis.

Ang II, like ET-1, is a peptide with mitogenic and angiogenic effects. In similar fashion to ET-1, it is generated by a proteolytic cascade, culminating in its production from angiotensin I (Ang I) by angiotensin converting enzyme (ACE), a key step in the renin-angiotensin system (RAS). ACE hydrolyses Ang I at the at the Phe-8-His-9 bond in order to produce Ang II. The RAS is made up of a number of different peptides and their specific receptors. Ang II is the main peptide effector of the RAS. Ang II can exert its effects by binding to two specific GPCRs; AT_1R and AT_2R. Ang II exerts most of its pathophysiological effects through the AT_1R (Fujiyama *et al*, 2001; Shah and Catt, 2003; Tamarat *et al*, 2002).

Evidence suggests that the local RAS may influence tumourigenesis by affecting tissue angiogenesis, inflammation, apoptosis and cellular proliferation (Deshayes and Nahmias, 2005; Juillerat-Jeanneret *et al*, 2004; De Paepe *et al*, 2001; Uemura *et al*, 2003). The expression of different components of the RAS are frequently known to undergo change in cancers in comparison to non-malignant tissue. This is the case in breast, prostate, lung, skin, colon and cervical cancers (Deshayes and Nahmias, 2005) and the alterations in expression levels often correlate with poor patient outcomes (George *et al*, 2010). The expression of the AT_1R is frequently increased in a number of these malignancies including breast cancer (Ager *et al*, 2008). Components of the RAS system have also been linked with tumour grading however no clear correlation has been observed and it very much depends on the tumour type as to how the components vary in malignant tissue compared to normal tissue (Louis *et al*, 2007; Sitzmann *et al*, 1994). Studies first identified a link between the RAS and tumourigenesis when patients who were administered ACE inhibitors as a long term treatment seemed to be protected against cancer (George *et al*, 2010). Further studies have identified that antagonism of the RAS resulted in the suppression of angiogenesis, tumour growth and metastasis (George *et al*, 2010).

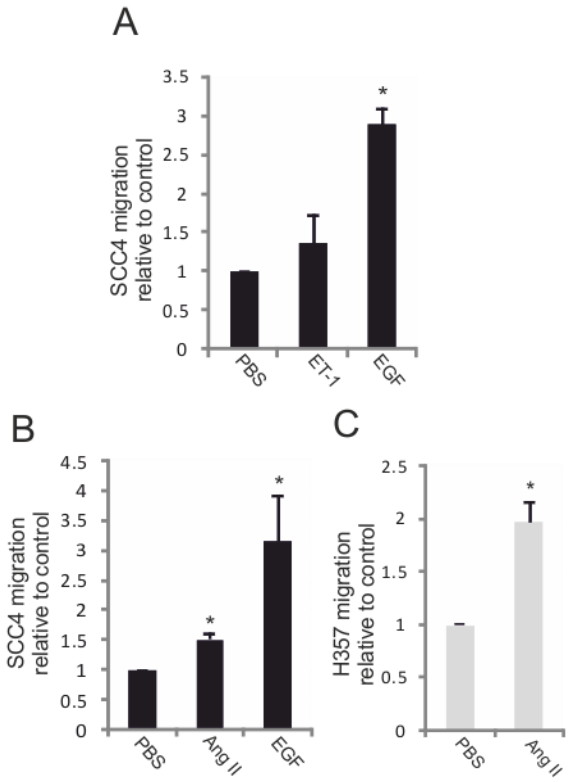


Figure 3.1 Ang II but not ET-1 stimulates HNSCC cells to migrate: Migration of SCC4 cells when treated with ET-1 (10 nM) or EGF (48.4 µM) **(A)** and Ang II (100 nM) or EGF (48.4 µM) **(B)** and of another HNSCC cell line H357 when treated with Ang II **(C)** were assayed using a 2D Transwell migration assay. Briefly, $1x10^5$ serum starved cells treated as above were added to the top of the Transwell migration inserts (8 µm pore) in DMEM supplemented with 0.1% (w/v) BSA with DMEM containing 10% (v/v) FBS placed at the bottom of the well. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. Standard error mean (± SEM) are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*).

Nothing is known to date, however, of the effect of ET-1 and Ang II on the chemotaxis and invasion of HNSCC cells, key steps in the metastatic cascade.

3.2 Ang II, but not ET-1, stimulates migration and invasion of HNSCC cells

Here, the ability of the mitogenic peptide ET-1 to promote SCC4 (an epithelial HNSCC cell line isolated from the tongue; Table 2.2) cell migration *in vitro* was investigated. SCC4 cells which had been serum starved for the previous 24 h were treated directly with ET-1, bradykinin (BK) or a phosphate buffered saline (PBS) vehicle control and their ability to migrate was assessed using a 2D Transwell migration assay. The Transwell insert used is composed of a permeable membrane containing 8 μm pores. DMEM containing 10% FBS was added to the bottom well of the Transwell migration assay, into which the insert sits. The cells were allowed to migrate through the permeable membrane for 16 h. Having migrated through the pores the cells adhere to the underside of the insert. These migrated cells were fixed, stained counted (as previously described in Section 2.2.4).

The treatment of SCC4 cells with ET-1 resulted in a small increase in cellular migration (1.3-fold) in comparison to PBS vehicle treated cells (Figure 3.1A). This increase in SCC4 cell migration did not reach statistical significance ($p=0.13$). The increase in migration was not to the same level as that observed when the cells were treated with BK, another mitogenic peptide known to promote HNSCC cell migration via an autocrine mechanism (Thomas *et al*, 2006). BK stimulated SCC4 migration 3-fold in comparison to vehicle treated cells (Figure 3.1A).

The ability of Ang II to promote SCC4 cell migration was investigated using the same Transwell migration assay described previously. Treatment of SCC4 cells with Ang II resulted in a significant increase of 1.5-fold in cell migration compared to PBS vehicle treated cells (Figure 3.1B). Treatment of SCC4 cells with EGF, a known stimulant of cancer cell motility via EGFR activation (Gullick, 1991), was used as a positive control and resulted in a significantly higher increase in migration. EGF stimulated SCC4 cells to migrate 3.0-fold compared to PBS vehicle treated cells. This increase in HNSCC migration provoked by Ang II was also observed to a greater extent when H357 cells (another epithelial HNSCC cell line isolated from the tongue; Table 2.2) were treated with the mitogenic peptide (2.0- fold vs. 1.5-fold for H357 and SCC4 cells, respectively), suggesting that this observation is not cell-line specific (Figure 3.1C). The results suggest that Ang II is able to directly stimulate HNSCC migration.

Having established that Ang II stimulates the ability of HNSCC cells to traverse a synthetic basement membrane, its ability to induce their invasion was next investigated. SCC4 and H357

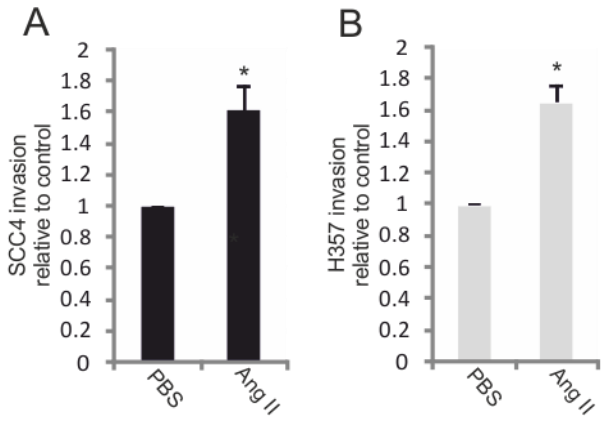


Figure 3.2 Ang II stimulates HNSCC cells to invade: Invasion of SCC4 cells **(A)** and H357 cells **(B)** when treated with Ang II (100 nM) were assayed using a Matrigel invasion assay. Matrigel, a murine tumour-derived extracellular matrix substitute was, coated onto invasion inserts and incubated for 24 h. Briefly, $1x10^5$ serum starved cells treated as above were added to the top of the invasion inserts in DMEM supplemented with 0.1% BSA (w/v) with DMEM containing 10% (v/v) FBS placed at the bottom of the well. After 40 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Invaded cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which invaded relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*).

cells were treated with Ang II and their ability to invade was assessed using a 2D Matrigel invasion assay. A layer of Matrigel, a murine tumour-derived extracellular matrix substitute, was added into the top of the migration insert. Matrigel is commonly used to measure the ability of cells to invade (Lang *et al*, 2000). The invasion of cancer cells through the basement membrane and the extracellular matrix (ECM) is a critical step in the process of metastasis. Matrigel contains a number of ECM components and therefore helps to create an environment *in vitro* more similar to that observed *in vivo*. The Matrigel was added to the top of the Transwell insert and allowed to set overnight. The following day SCC4 cells or H357 cells were treated with Ang II and were placed into the insert. After 40 h cells were fixed, stained and counted as described in Section 2.2.5. An increase in cellular invasion was observed in both cell lines of a similar magnitude to that observed for migration (1.6-fold increase for both SCC4 cells and H357 cells) (Figure 3.2A and 3.2B for SCC4 and H357 cells, respectively).

3.3 Ang II stimulates an increase in HNSCC migration and invasion through the AT_1R

The mechanism by which Ang II stimulates HNSCC cells to migrate and invade was next examined. Ang II can exert its effects by binding to two specific GPCRs, AT_1R and AT_2R. Ang II exerts most of its pathophysiological effects via AT_1R, which is expressed on a number of different cell types. These effects include increased cell proliferation, production of growth factors and cytokines and increased fibrosis, which can lead to vascular thickening and atherosclerosis (Fujiyama *et al*, 2001; Shah and Catt, 2003; Tamarat *et al*, 2002). The AT_2R is mainly expressed during early stages of foetal development, however it is also expressed in the adrenal medulla, ovarian follicles and the uterus during an adult's lifetime (Messerli *et al*, 1996; Touyz *et al*, 2000; Velasquez, 1996). It has been suggested that the receptor can antagonise proliferation, angiogenesis and inflammatory responses that are activated via the AT_1R (Pupilli *et al*, 1999).

qPCR was used to determine the expression pattern of both the AT_1R (Figure 3.3A) and AT_2R (Figure 3.3B) in a panel of primary human normal oral keratinocytes (NOKs) and fibroblasts (NOFs) and cell lines derived from oral dysplasias, primary HNSCCs and a local metastasis. The expression of AT_1R was marked elevated in the primary HNSCC cell lines, which included Cal27s (an epithelial HNSCC cell line isolated from the tongue; Table 2.2), H357s, SCC4s and B16s (an epithelial HNSCC cell line isolated from the tongue; Table 2.2), in comparison to the NOKs and the cell lines D19 and D20, both of which are derived from oral dysplasias and the cell line B22, which was isolated from a local metastasis, all of which are described in Table 2.2. Attempts were made to detect AT_1R by western blot analysis but were unsuccessful. There

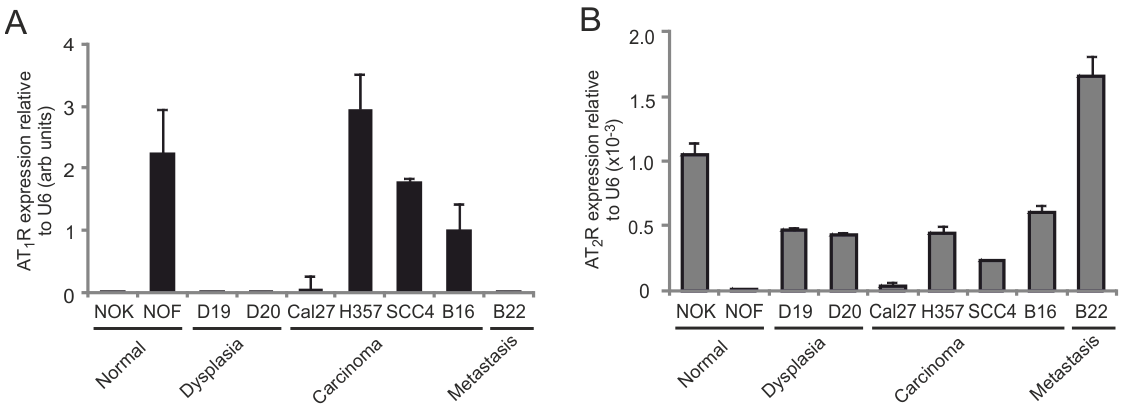


Figure 3.3 AT_1R is largely over expressed in HNSCC cell lines: Primary human normal oral keratinocytes (NOKs) and fibroblasts (NOFs) and cell lines derived from oral dysplasias, primary HNSCCs and a local metastasis were cultured in growth media and at 90% confluency were washed in PBS, trypsinised and pelleted. Total RNA was extracted from the cell pellets and 100 ng was subjected to RT-PCR. 0.25 ng cDNA was analysed using qPCR for AT_1R **(A)**, AT_2R **(B)** or U6 as a reference gene. Each data point represents an average of 3 technical repeats. ± SEM are indicated.

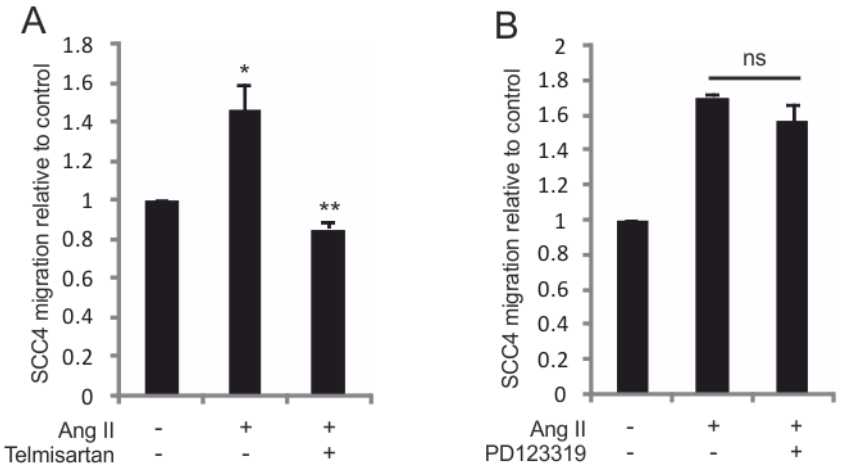


Figure 3.4 Ang II exerts its effects through the AT_1R: To determine which receptor Ang II was exerting its effects via, SCC4 cells were pre-treated with a specific receptor antagonist to AT_1R, telmisartan (100 µM) **(A)** and a specific receptor antagonist to AT_2R, PD123319 (100 µM) **(B)** for 30 min before the addition Ang II (100 nM) and were assayed using a 2D Transwell migration assay. Briefly, $1x10^5$ serum starved cells treated as above were added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% BSA (w/v) with DMEM containing 10% (v/v) FBS placed at the bottom of the well. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p < 0.05$ relative to Ang II treated cells (**), ns = not significant.

was no obvious pattern in the transcript levels of the AT_2R within the panel of NOKs and cell lines derived from the oral dysplasias, primary HNSCCs and a local metastasis. There was a slight trend towards the down-regulation of AT_2R in primary HNSCC cell lines in comparison to NOKs and other cell lines. There was no expression of the AT_2R detectable within the NOFs. To determine which of these receptors are involved in the autocrine stimulation of HNSCC migration and invasion, SCC4 cells were pre-treated with specific receptor antagonists to either the AT_1R (Figure 3.4A) or the AT_2R (Figure 3.4B) for 30 min before the addition of the Ang II peptide. Pre-treatment of SCC4 cells with the AT_1R antagonist, telmisartan, blocked stimulation of cellular migration induced by Ang II. Migration was reduced from 1.5-fold in comparison to PBS vehicle treatment to 0.8-fold in the presence of the telmisartan. No difference in migration was observed when SCC4 were pre-treated with the AT_2R antagonist, PD123319, the fold increase remained at 1.6-fold. This observation suggests that Ang II is exerting its effects through AT_1R.

3.4 The over expression of ACE can stimulate HNSCC migration

ACE is a zinc-metalloproteinase and is responsible for the production of Ang II from the biologically inactive precursor Ang I, which is a key step in the proteolytic cascade regulating the RAS. Somatic ACE is expressed on the surface of endothelial and epithelial cells in a wide variety of tissues. qPCR was used to determine the transcript levels of ACE within a panel of primary human NOKs and NOFs and cell lines derived from oral dysplasias, primary HNSCCs and a local metastasis as described in Table 2.2 (Figure 3.5A). ACE transcript levels were detectable in all primary cells and cell lines. The enzyme was greatly over expressed in NOFs. The effect of over expressing ACE on SCC4 migration was next examined. SCC4 cells were transiently transfected using FuGENE 6 with a pIRES vector containing the somatic ACE coding region or a control pcDNA3 vector for 24 h. After 24 h the transfected cells were trypsinised, counted and added to the Transwell insert. DMEM containing 10% FBS was added to the bottom well of the Transwell migration assay, into which the insert was placed. The cells were allowed to migrate through the permeable membrane for 16 h. Once through the pores the cells were fixed, stained and counted as described in Section 2.2.4. Over expression of ACE resulted in an increase in SCC4 migration of 1.5-fold in comparison to cells transfected with a control plasmid (Figure 3.5B). qPCR analysis was conducted on the remaining SCC4 cells to determine the expression level of ACE after transfection (Figure 3.5C). The expression of ACE increased 212.0-fold in SCC4 cells after transfection with the pIRES plasmid containing the coding region for somatic ACE in comparison to SCC4 cells transfected with the control pcDNA3 vector.

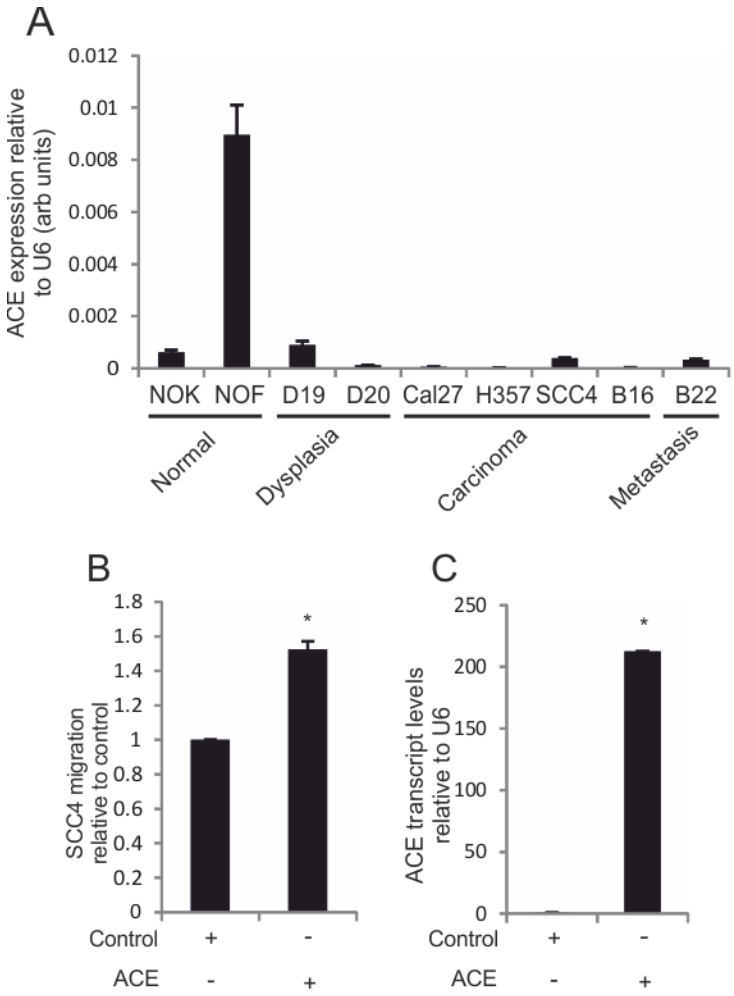


Figure 3.5 Over expression of ACE in SCC4 cells increases their migration capability: ACE is a zinc-metalloproteinase and is responsible for the production of Ang II from Ang I. Primary human NOKs and NOFs and cell lines derived from oral dysplasias, primary HNSCCs and a local metastasis were cultured in growth media and at 90% confluency were washed in PBS, trypsinised and pelleted. Total RNA was extracted from the cell pellets and 100 ng was subjected to RT-PCR. 0.25 ng cDNA was analysed using qPCR for ACE (A) or U6 as a reference gene. SCC4 cells were transiently transfected with an ACE vector or control vector for 24 h before they were added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA and DMEM containing 10% (v/v) FBS placed at the bottom of the well. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope **(B)**. Transfected SCC4 cells were harvested and RNA extracted. qPCR analysis was used to measure the transcript levels of ACE **(C)**. Each qPCR data point represents an average of 3 technical repeats. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p<0.05$ relative to control transfected cells.

3.5 Components of the ET-axis can influence HNSCC cell migration in an autocrine manner

Although ET-1 resulted in only a small increase in SCC4 migration (1.3-fold) (Figure 3.1A), reports of elevated levels of ET-1 in HNSCC prompted the examination of transcript levels of components of the ET axis in cells derived from normal and diseased oral mucosa. qPCR was used to determine the transcript levels of ET-1 within a panel of primary human NOKs and NOFs and cell lines derived from oral dysplasias, primary HNSCCs and a local metastasis as described in Table 2.2 (Figure 3.6A). ET-1 transcript was present in all cell types but no significant pattern was observed. ECE-1 converting enzyme is responsible for the catalytic activation of ET-1. ECE-1 cleaves big ET to release biologically active ET-1. Neprilysin (NEP) is responsible for the inactivation of a number of physiologically active neuropeptides including ET-1, BK, neurotensin, atrial natriuretic factor and substance P (Shipp *et al*, 1991; Shipp and Look, 1993). ECE-1 and NEP are both zinc metalloproteases and are members of the M13 family and share 40% homology with each other (Lambert *et al*, 2008). qPCR was again used to determine the transcript levels of both ECE-1 (Figure 3.6B) and NEP (Figure 3.6C) in the same panel of cells. ECE-1 was highly expressed in NOFs and all cell lines apart from H357s which only showed a slightly greater expression in comparison to NOKs. NEP was highly expressed in NOFs and was down regulated in the primary HNSCC cell lines. qPCR was used to determine the transcript levels of ET_AR (Figure 3.7A) and ET_BR (Figure 3.7B) within the same cells and cell lines. ET_AR transcript levels were highly expressed in NOFs and were present in all other cell types. ET_BR transcript was also readily detectable in NOFs and was reduced in HNSCC cell lines and the local metastasis, B22. This is in keeping with previous findings that the receptor is reduced in HNSCC cell lines (Okawawa *et al*, 1998; Viet *et al*, 2011).

3.6 ECE-1 protein expression in cell lines

As mentioned previously ECE-1 is responsible for the production of biologically active ET-1. SDS-PAGE and western blot analysis was used to determine ECE-1 protein expression in the panel of primary human NOKs and NOFs and cell lines derived from oral dysplasias, primary HNSCCs and a local metastasis described in Table 2.2. ECE-1 was greatly overexpressed in NOFs and in all the cell lines in comparison to NOKs (Figure 3.8), this result was similar to that observed for qPCR analysis (Figure 3.6B).

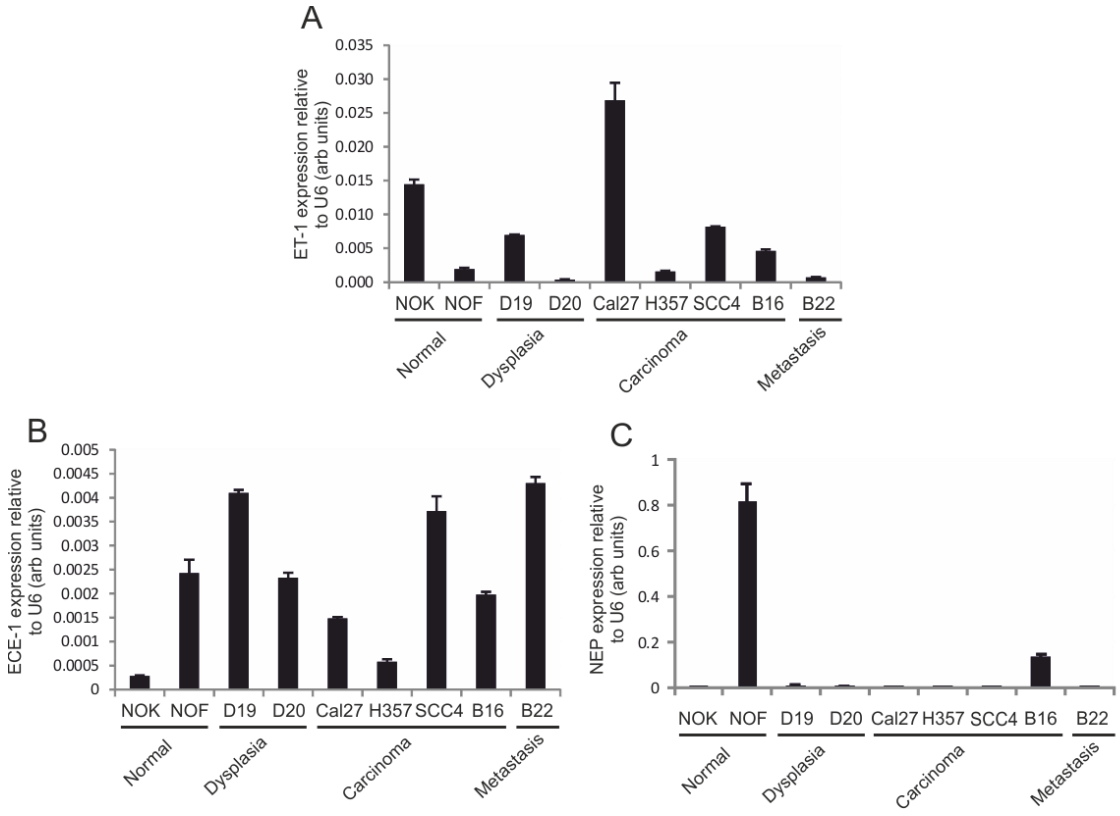


Figure 3.6 Components of the ET-axis are present in HSCCC cell lines: Primary human NOKs and NOFs and cell lines derived from oral dysplasias, primary HNSCCs and a local metastasis were cultured in growth media and at 90% confluency were washed in PBS, trypsinised and pelleted. Total RNA was prepared from the cell pellets and 100 ng was subjected to RT-PCR. 0.25 ng cDNA was analysed using qPCR for ET-1 **(A)**, ECE-1 isoforms **(B)**, NEP **(C)** or U6 as a reference gene. Each data point represents an average of 3 technical repeats. ± SEM are indicated.

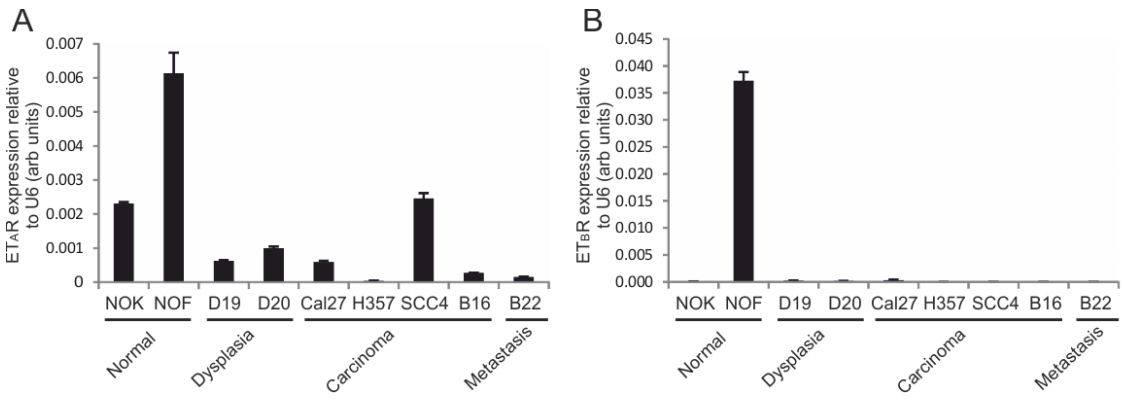


Figure 3.7 ETAR and ETBR expression: ET-1 can exert its cellular actions by binding to one, or both, of two GPCRs, ETAR and ETBR. Primary human NOKs and NOFs and cell lines derived from oral dysplasias, primary HNSCCs and a local metastasis were cultured in growth media and at 90% confluency were washed in PBS, trypsinised and pelleted. Total RNA was prepared from the cell pellets and 100 ng was subjected to RT-PCR. 0.25 ng cDNA was analysed using qPCR for ETAR **(A)**, ETBR **(B)** or U6 as a reference gene. Each data point represents an average of 3 technical repeats. ± SEM are indicated.

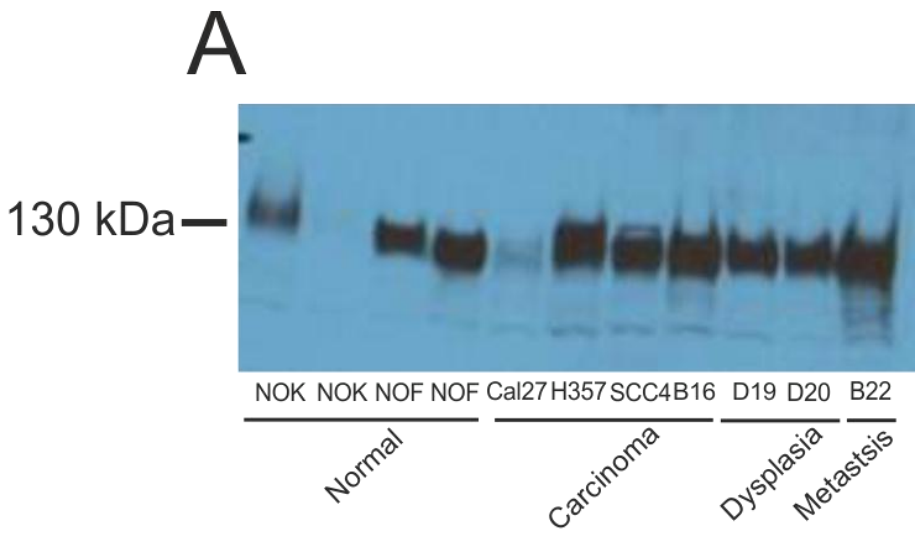


Figure 3.8 ECE-1 protein expression: Total protein was extracted from a panel of primary human NOKs and NOFs and cell lines derived from primary HNSCCs, oral dysplasias and a local metastasis and 50 μg separated by SDS-PAGE and immunoblotted for ECE-1. A representative blot is shown **(A)**.

3.7 The over expression of ECE-1c can stimulate HNSCC migration

ECE-1 has four distinct isoforms: ECE-1a, ECE-1b, ECE-1c and ECE-1d. All four isoforms are derived from a single gene using alternative promoter regions. ECE-1a and ECE-1c are both localised at the cell surface and ECE-1b and ECE-1d are found within intracellular compartments. Each isoform has a different N-terminal cytoplasmic region and it is this characteristic that is responsible for the targeting of each isoform to its correct cellular location (Muller *et al*, 2003). ECE-1c is the most abundant ECE-1 isoform and its expression is elevated significantly in a number of tumours including prostate (Muller *et al*, 2003). SCC4 cells were transiently transfected using FuGENE 6 with a pcDNA3 vector containing the ECE-1c coding region or a control pcDNA3 vector for 24 h. After 24 h cells were trypsinised, counted and added to the Transwell insert. DMEM containing 10% FBS was added to the bottom well of Transwell migration assay, into which the insert sits. The cells were allowed to migrate through the permeable membrane for 16 h. The cells were fixed, stained and counted as described in Section 2.2.4 (Figure 3.9A). Over expression of ECE-1c resulted in a 1.4-fold increase in SCC4 migration in comparison to cells transfected with the control pcDNA3 vector. qPCR analysis was conducted on the remaining SCC4 cells to determine ECE-1 expression and therefore transfection efficiency (Figure 3.9B). The expression of ECE-1 increased 9-fold in SCC4 cells after transfection with the pcDNA3 vector containing the ECE-1c coding region in comparison to SCC4 cells transfected with the control pcDNA3 vector.

3.8 Inhibition of ECE-1 does not reduce HNSCC migration

A broad range inhibitor to all four ECE-1 enzyme isoforms was used to determine if inhibiting the enzyme could reduce SCC4 cellular migration. SCC4 cells were treated with SM-19712 before their addition to the Transwell insert. The presence of the inhibitor did not reduce SCC4 migration.

3.9 Summary

In this chapter the ability of ET-1 and Ang II to promote HNSCC migration via an autocrine mechanism was investigated. Although ET-1 treatment of HNSCC cells resulted in a small increase in migration, this was not deemed statistically significant. However recent studies have observed elevated levels of the mitogenic peptide in HNSCC cell lines and oral specimens (Awano *et al*, 2006; Pickering *et al*, 2007) and it was therefore deemed necessary to determine the transcript levels of components of the ET-axis within a panel of primary cells and cell lines. Notably ECE-1, the enzyme responsible for the activation of biologically active ET-1 was over expressed in nearly all the cell lines. The transient transfection of a pcDNA3

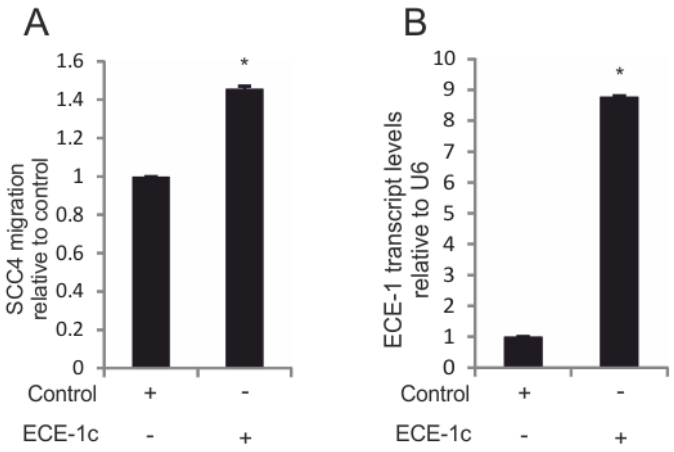


Figure 3.9 Over expression of ECE-1c in SCC4 cells increases their migration capability: ECE-1 is a membrane bound metalloproteinase and is responsible for the proteolytic cleavage of big ET-1 to ET-1 between Trp-21-Val/Ile-22 bond. This event results in biologically active ET-1. ECE-1 has four distinct isoforms: ECE-1a, ECE-1b, ECE-1c and ECE-1d. The expression of ECE-1c is elevated in a number of tumours including prostate cancer (Muller *et al*, 2003). SCC4 cells were transiently transfected with a pcDNA3 vector containing the ECE-1c coding region or control pcDNA3 vector for 24 h before they were added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA and DMEM containing 10% (v/v) FBS placed at the bottom of the well. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope **(A)**. Transfected SCC4 cells were harvested and RNA extracted. qPCR analysis was used to measure the transcript levels of ECE-1c **(B)**. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p<0.05$ relative to control transfected cells.

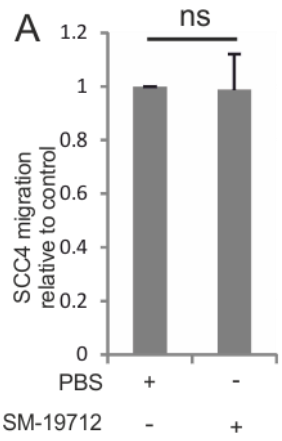


Figure 3.10 ECE-1 inhibition does not block SCC4 migration: ECE-1 is known to cleave biologically active ET-1 from big ET-1. SCC4 cells were treated with an ECE-1 inhibitor, SM-19712 (10 μM) **(A)** before they were added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA and DMEM containing 10% (v/v) FBS placed in the bottom of the well. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), ns = not significant.

vector containing the ECE-1c coding region into SCC4 resulted in a significant increase in their migration. The previous findings in this chapter suggest that the production of ET-1 by ECE-1 is not responsible for this increase in HNSCC migration. ECE-1 is known to hydrolyse and affect the activity of other peptides which could explain the increase in HNSCC migration.

Ang II unlike ET-1 can stimulate HNSCC migration and invasion via an autocrine mechanism. This chapter has identified that Ang II in this case exerts its effects through the AT_1R which is largely over expressed in primary HNSCC cell lines, implicating the receptor as a valuable diagnostic tool or possible therapeutic target in the treatment of HNSCC. When SCC4 cells were transfected with a pIRES vector containing the coding region for somatic ACE, their ability to migrate increased. This increase in HNSCC migration was to a similar extent as that observed when the cells were treated directly with the peptide therefore suggesting that the over expression of the ACE enzyme results in the production of more Ang II which can act on the SCC4 cells in an autocrine fashion stimulating their migration.

Chapter 4: The effect of mitogenic peptides on paracrine signalling in head and neck cancer progression

4.1 Introduction

It is becoming increasingly apparent that the development and progression of epithelial tumours is profoundly influenced by the surrounding microenvironment, which is also known as the reactive stroma. The tumour microenvironment consists of a number of different cellular components including fibroblasts, blood vessels and lymphatics, extracellular matrix (ECM) components and immune and inflammatory cells. Any or all of these stromal components can interact and communicate with cancer cells and influence their behaviour. Communication can also occur between different cells and cell types found within the reactive stroma.

Studies have identified that endothelin-1 (ET-1) can be produced by a number of epithelial tumours including those of the pancreas, breast, lung, prostate, colon and endometrium (Bagnato and Catt, 1998). The peptide is also produced by a number of cancer cell lines cultured *in vitro*. These cell lines include those isolated from carcinomas of the stomach, prostate, breast and colon (Bagnato and Catt, 1998). Lambert *et al* (2008) have identified that elevated levels of ET-1 are associated with metastatic prostate cancer and have been implicated in the cross talk that occurs between tumour and stromal cells. This cellular communication promotes autocrine and paracrine signalling between the different cell types resulting in an increase in tumour growth. Deregulation of the renin angiotensin system (RAS), including an increase in angiotensin II (Ang II) production has been identified in malignant tissue and also within the surrounding tumour microenvironment (George *et al*, 2010). The RAS in the reactive stroma has been associated with an increase in vascular endothelial growth factor (VEGF) production and therefore increased angiogenesis (George *et al*, 2010).

Both lines of evidence highlight that increased levels of mitogenic peptides can be found within the microenvironment. The role that mitogenic peptides, including ET-1 and Ang II, play within this environment and the effect that they have on the components found within it are not fully elucidated. Fibroblasts are the most numerous cell type found within the reactive stroma and those found within this tumour microenvironment frequently undergo changes in their phenotype. It was therefore decided to examine the role of primary normal oral fibroblasts (NOFs) in mediating the effects of ET-1 and Ang II on head and neck squamous cell carcinoma (HNSCC) behaviour.

4.2 NOFs potentiate the effect of ET-1 and Ang II on HNSCC motility

In order to investigate the effect of the presence of NOFs on HNSCC migration, NOFs were seeded into the bottom of a 2D Transwell migration assay. SCC4 cells and the seeded cells were treated with ET-1 and the ability of the SCC4 cells to migrate was assessed in the

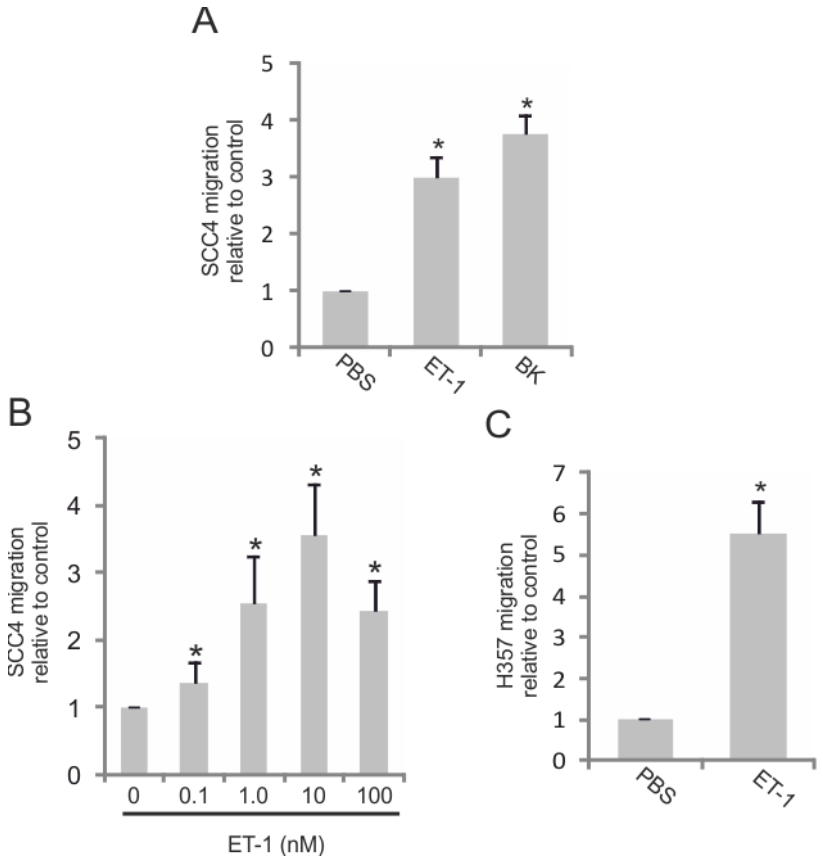


Figure 4.1 NOFs potentiate the effect of ET-1 on HNSCC migration: The tumour microenvironment plays a role in the progression of epithelial tumours. To create an environment more similar to that observed *in vivo,* NOFs were seeded into the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells were treated with ET-1 (10 nM) or BK (1 μM) and added to the top of the Transwell migration inserts (8 μm pore) in DMEM supplemented with 0.1% (w/v) BSA **(A)**. The presence of the NOFs within the assay potentiated the effect of ET-1 on SCC4 cell migration. To determine the signalling mechanism involved in this paracrine activation, NOFs were treated with ET-1 (0 – 100 nM) for 4 h before the media was aspirated, filtered and added to the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% BSA **(B)**. NOFs were treated with the optimal concentration of ET-1, 10 nM and the conditioned media was added to the bottom Transwell migration well. $1x10^5$ serum starved H357 cells were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% BSA (w/v) **(C)**. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. Standard error mean (± SEM) are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*).

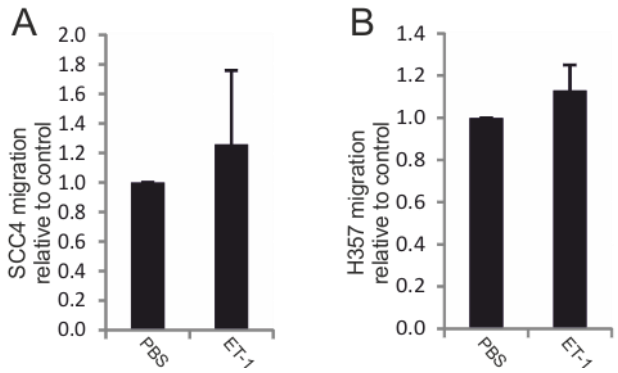


Figure 4.2 ET-1 does not act as a chemoattractant for HNSCC cells: To investigate the paracrine effect of ET-1 on HNSCC cell migration, ET-1 (10 nM) in serum free media was added to the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells **(A)** or H357 cells **(B)** were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*).

presence of the NOFs. The presence of NOFs dramatically enhanced the effect of ET-1 on SCC4 cellular migration (3.0- vs. 1.3-fold in the presence and absence of NOFs, respectively; Figure 4.1A and Figure 3.1A). The presence of NOFs within the 2D migration model imitates an environment more similar to that that would be observed *in vivo*. In contrast, inclusion of NOFs had little effect on the stimulation of SCC4 migration by bradykinin (BK) (3.8- vs. 3.3-fold in presence and absence of NOFs, respectively; Figure 4.1A and Figure 3.1A), suggesting BK acts in an autocrine manner, in keeping with previous observations (Thomas *et al*, 2006).

The presence of the NOFs within the migration assay potentiates the effects of ET-1 on HNSCC migration suggesting that the presence of the NOFs results in an increased release of soluble factors from the cells which could contribute to the ET-1-mediated stimulation of SCC4 cell migration. To determine the mechanisms involved in the paracrine activation of NOFs a system was designed in order to probe the signalling pathways concerned. NOFs were treated with varying concentrations of ET-1 (0-100 nm) for 4 h before the media was removed, filtered and added to the bottom of the Transwell migration assay. Serum starved SCC4 cells were left untreated and added to the migration insert. The cells were allowed to migrate for 16 h and were fixed, stained and counted using the same methods described previously (Section 2.2.4) (Figure 4.1B). The conditioned media collected from the NOFs stimulated SCC4 migration in a dose responsive manner. 0.1 nM ET-1 treatment resulted in a 1.5-fold increase in SCC4 migration, 1 nM ET-1 stimulated a greater increase of 2.5-fold. 10 nM ET-1 stimulated the optimal increase of 3.5-fold. 100 nM ET-1 treatment did not further increase SCC4 migration and instead migration decreased to only 2.4-fold. These observations suggest that ET-1 may saturate its receptor (s) on the surface of the NOFs at concentrations above 10 nM (Ferguson, 2001). The dose response observed suggests that soluble factors released by the NOFs into the surrounding media as a result of ET-1 stimulation are responsible for the paracrine activation of SCC4 cells. To determine if this paracrine effect was a general HNSCC phenomenon, rather than cell line specific, NOFs were treated with the optimal concentration of ET-1, 10 nM for 4 h. Instead of using SCC4 cells, serum starved H357 cells were left untreated and placed into the Transwell insert. An increase in migration of 5.0-fold was observed for the H357 cells suggesting that this observation is not cell-line specific (Figure 4.1C). The presence of 10 nM ET-1 alone, in the bottom of the Transwell migration assay, had no significant effect on HNSCC migration in the absence of NOFs (Figure 4.2A and 4.2B).

Unlike ET-1, Ang II is able to stimulate HNSCC cell migration and invasion via an autocrine mechanism (Section 3.2). Transwell migration assays were used to determine if Ang II can also stimulate SCC4 and H357 cells via a paracrine mechanism, similar to that observed for ET-1.

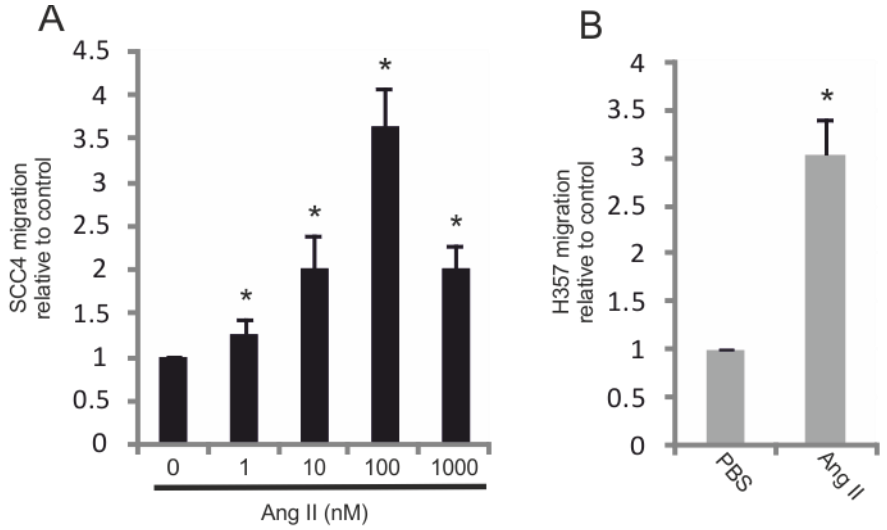


Figure 4.3 NOFs potentiate the effect of Ang II on HNSCC migration: To investigate the effect of NOFs on HNSCC cell migration, NOFs were treated with Ang II (0 – 1000 nM) for 4 h before the media was aspirated, filtered and added to the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA **(A)**. NOFs were treated with the optimal concentration of Ang II, 100 nm and the conditioned media was added to the bottom Transwell migration well. $1x10^5$ serum starved H357 cells were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA **(B)**. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*).

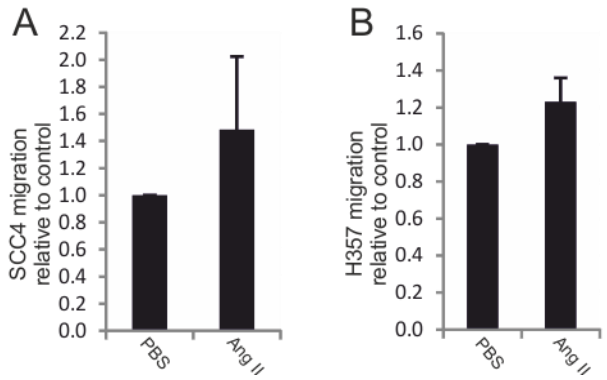


Figure 4.4 Ang II alone does not stimulate HNSCC cell line migration via a paracrine mechanism: To investigate the paracrine effect of Ang II on HNSCC cell migration, Ang II (100 nM) in serum free media was added to the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells **(A)** or H357 cells **(B)** were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA (A). After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*).

NOFs were treated with varying concentrations of Ang II (0-1000 nM) for 4 h and the conditioned media collected was added to the bottom of the Transwell migration well. The effect on SCC4 migration was observed. The conditioned media collected from the NOFs stimulated SCC4 migration in a dose responsive manner (Figure 4.3A). 1 nM Ang II treatment resulted in a 1.3-fold increase in SCC4 migration, 10 nM Ang II stimulated a higher fold increase of 2.0. 100 nM Ang II stimulated the optimal increase of 3.5-fold. 1000 nM Ang II treatment did not further increase SCC4 migration and instead migration decreased to only 2.0-fold. These observations suggest that Ang II may saturate its receptor (s) on the surface of NOFs at concentrations above 100 nM (Ferguson, 2001). Again, the presence of NOFs dramatically enhanced the effect of the Ang II on SCC4 cellular migration (3.5- vs. 1.5-fold in the presence and absence of NOFs, respectively; Figure 4.3A and Figure 3.1B). The results indicate that Ang II does stimulate the release of soluble factors from NOFs into the surrounding environment and that these can trigger pro-migratory paracrine signalling between the NOFs and cancer cells, a similar mechanism to that triggered by ET-1 stimulation. It was next determined if the effect observed on SCC4 cells was cell line specific, or would also be seen in other HNSCC-derived cells. NOFs were treated with 100 nM Ang II for 4 h before the media was aspirated, filtered and added to the Transwell migration well. Serum starved H357 cells were left untreated before they were added to the Transwell insert and allowed to migrate (Figure 4.3B). The presence of the conditioned media resulted in a fold increase of 3.0- in comparison to NOFs incubated with PBS alone. Therefore it was apparent that the migration of HNSCC cells was dramatically enhanced in the presence of NOF conditioned media (3.5- vs. 1.5-fold in the presence and absence of conditioned media, respectively, for SCC4 and 3.0- vs. 1.8-fold in the presence and absence of conditioned media, respectively, for H357). The presence of Ang II alone, in the lower chamber of the Transwell migration assay, resulted in a small but insignificant increase in HNSCC migration and invasion in the absence of NOFs (Figure 4.4A and 4.4B).

4.3 NOFs potentiate the effect of ET-1 and Ang II on HNSCC invasion

Having demonstrated that both ET-1 and Ang II can promote HNSCC cell migration via the activation of paracrine signalling mechanisms between cancer cells and stromal NOFs, the effect of conditioned media on cellular invasion was investigated. The ability of ET-1 to promote SCC4 invasion via a similar paracrine mechanism to that observed for HNSCC migration was investigated. Matrigel is a murine-derived ECM substitute that is commonly used to measure the ability of cells to invade. The ability of cancer cells to invade into and through the ECM is crucial in the metastasis process. Matrigel was diluted 1:45 (v/v) in serum

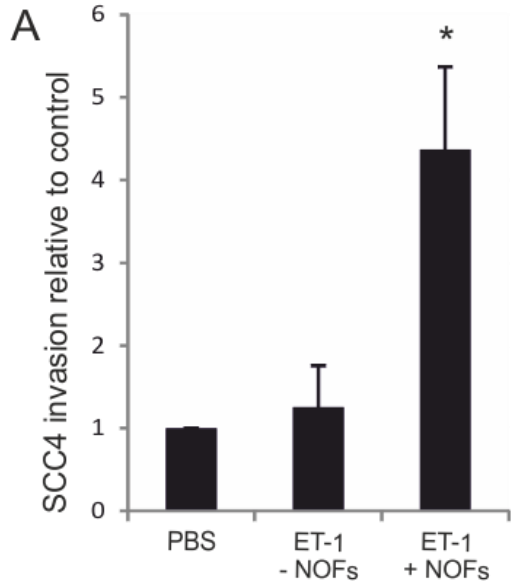


Figure 4.5 NOFs potentiate the effect of ET-1 on HNSCC invasion: The effect of NOFs on SCC4 cell invasion was investigated. Matrigel, a murine tumour-derived extracellular matrix substitute was coated onto invasion inserts and incubated for 24 h. Conditioned media collected from NOFs treated with 10 nM ET-1 for 4 h serum free media treated with 10 nM ET-1 II was added to the bottom Matrigel invasion well. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Matrigel invasion inserts in DMEM supplemented with 0.1% (w/v) BSA **(A)**. After 40 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Invaded cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*).

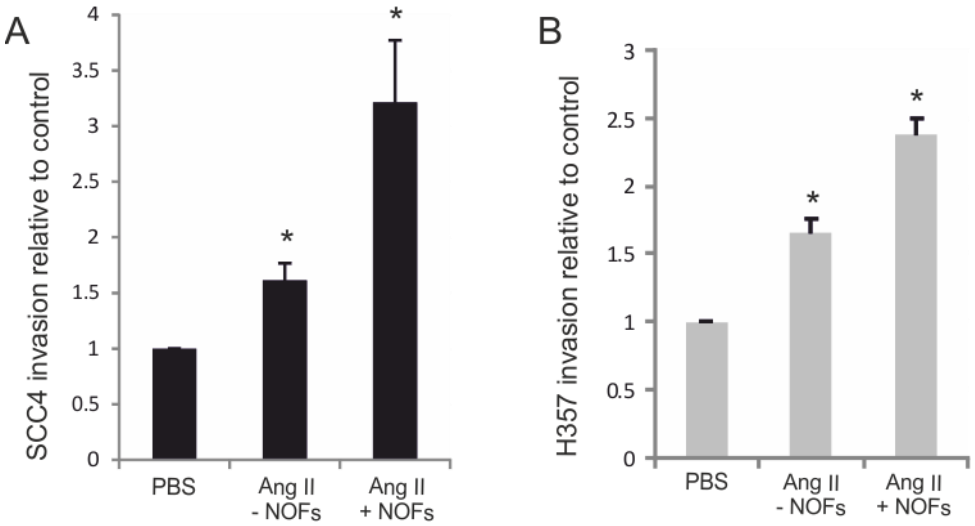


Figure 4.6 NOFs potentiate the effect of Ang II on HNSCC invasion: The effect of oral fibroblasts on HNSCC cell invasion was investigated. Matrigel was coated onto invasion inserts and incubated for 24 h. Conditioned media collected from NOFs treated with 100 nM Ang II for 4 h or serum free media treated with 100 nM Ang II was added to the bottom Matrigel invasion well. $1x10^5$ serum starved SCC4 cells **(A)** or H357 cells **(B)** were left untreated and added to the top of the Matrigel invasion inserts in DMEM supplemented with 0.1% (w/v) BSA. After 40 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Invaded cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*).

free DMEM. 100 µl of diluted Matrigel was added to each invasion insert and allowed to set overnight. The invasion assay was then undertaken using a similar experimental procedure as that used to test HNSCC migration. Treatment of NOFs with ET-1 resulted in a similar increase of 4-fold in invasion of SCC4 cells to that observed in the previous migration experiment (Figure 4.5A). This was in comparison to conditioned media collected from PBS vehicle treated NOFs.

The ability of Ang II to promote both SCC4 and H357 invasion was also examined. Conditioned media collected from NOFs treated with Ang II stimulated an increase in SCC4 cellular invasion of 4-fold and an increase in H357 cellular invasion of 2.4-fold, both in comparison to conditioned media collected from PBS treated NOFs (Figure 4.6A and 4.6B).

4.4 ET-1 exerts its paracrine effects through both ET_AR and ET_BR

The mechanism by which ET-1 stimulates NOFs to promote HNSCC migration and invasion was next examined. ET-1 can exert its cellular actions by binding to one or two or both GPCRs, ET_AR and ET_BR. To examine which of these receptors was involved in the paracrine stimulation of HNSCC migration by ET-1 the expression pattern of both receptors in SCC4 and NOFs was determined. qPCR was conducted on primary cells and cell lines to establish transcript levels of both ET_AR and ET_BR (Section 3.5). Agarose gel analysis conducted on these samples confirmed that ET_AR was detectable in both SCC4 and NOFs but ET_BR was only detectable in NOFs (Figure 4.7A). In order to determine which receptor ET-1 was exerting its effects through, cells were pre-treated with a specific ET_AR antagonist, BQ-123 and a specific ET_BR antagonist, BQ-788 for 30 min before the addition of the ET-1 for a further 4 h. Serum starved SCC4 cells were left untreated and their ability to migrate in the presence of conditioned media collected from NOFs treated with each receptor antagonist was investigated (Figure 4.7B). The presence of both receptor antagonists significantly ablated SCC4 cellular migration stimulated by ET-1 treatment. Treatment of NOFs with ET-1 alone resulted in a 3.0-fold increase in SCC4 migration. When the NOFs were pre-treated with an antagonist to ET_AR, BQ-123 this resulted in a decrease of SCC4 migration to only 1.4-fold relative to vehicle control. When the NOFs were pre-treated with an antagonist to ET_BR, BQ-788 this resulted in a decrease of SCC4 migration to 1.7-fold compared to control.

To determine which receptors are responsible for the paracrine stimulation of SCC4 invasion, a similar experiment to that described in the previous paragraph was conducted. Conditioned media was collected from NOFs treated with ET-1 +/- receptor antagonists to ET_AR, BQ-123 or ET_BR, BQ-788. The media was added to the bottom well of a 2D Matrigel invasion assay. ET-1 treatment of NOFs alone resulted in an increase in SCC4 invasion of 4.0-fold (Figure 4.7C). Pre-

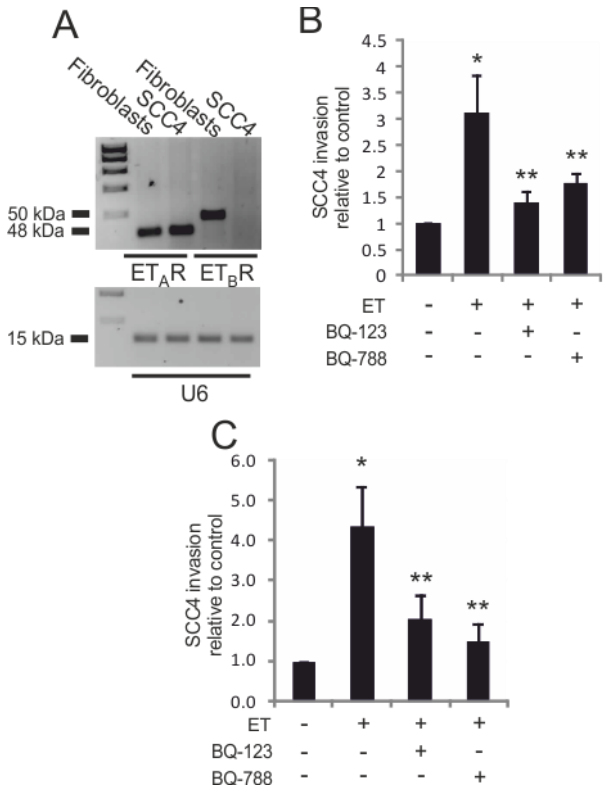


Figure 4.7 ET-1 mediates paracrine stimulation of HNSCC migration and invasion via ETAR and ETBR: To determine which receptor ET-1 exerts its paracrine effects via RNA was extracted from SCC4 and NOFs and subjected to qPCR for ETAR, ETBR or U6 as a reference gene. Amplicons were separated by agarose gel electrophoresis and visualised by UV transillumination **(A)**. OFs were pre-treated with a specific receptor antagonist to ETAR, BQ-123 (1 μM) or to ETBR, BQ-788 (1 μM) for 30 min before the addition ET-1 (10 nM) for a further 4 h. Conditioned media was aspirated, filtered and added to the bottom Transwell migration well **(B)** or the bottom Matrigel invasion well **(C)**. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration or Matrigel invasion inserts in DMEM supplemented with 0.1% (w/v) BSA. After 16 h (for migration) or 40 h (for invasion), cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p<0.05$ relative to ET-1 treated cells (**).

treatment of NOFs with BQ-123 caused a reduction in SCC4 invasion. Invasion was only 2.0-fold greater than that observed for untreated NOFs in comparison to the 4.0-fold increase that was observed when NOFs were only treated with ET-1. Pre-treatment of NOFs with BQ-788 resulted in a similar reduction in SCC4 invasion that was only 1.7-fold greater than that observed for NOFs that were left untreated. The blocking of ET-1 stimulated HNSCC invasion by the specific ET_AR antagonist, BQ-123 and the specific ET_BR antagonist, BQ-788 suggest that both receptors are involved in the paracrine signalling of NOFs.

4.5 Ang II exerts its paracrine effects through AT_1R

It is known that Ang II can exert its effect through binding to one or two or both GPCRs, AT_1R and AT_2R. As previously observed in Section 3.3 Ang II exerts its autocrine effects through the AT_1R which is markedly elevated in HNSCC primary carcinoma cell lines. The receptor is also expressed at readily detectable levels in NOFs (Figure 3.3A). The receptor, by which Ang II is exerting its effects through in NOFs, was next examined. Although the expression of AT_1R was detectable in NOFs, the expression of AT_2R was undetectable within the same cells (Figure 3.3B); it was therefore decided to examine the effect of antagonising AT_1R signalling on the responses observed to Ang II. The pre-treatment of NOFs with a specific antagonist to the AT_1R, telmisartan, resulted in the complete abrogation of Ang II-dependent SCC4 cellular migration and invasion in response to conditioned medium. NOFs were pre-treated with the receptor antagonist for 30 min before the addition of Ang II for a further 4 h. The effect of the antagonist on SCC4 migration and invasion in response to conditioned medium was determined by using a Transwell migration and a Matrigel invasion assay, respectively. When NOFs were treated with Ang II alone it resulted in an increase in SCC4 migration of 2.5-fold in comparison to vehicle treated cells (Figure 4.8A). This increase in migration was reduced to only 1.2-fold when telmisartan was present. Ang II treated NOFs stimulated SCC4 cells to invade 3.0-fold when compared to vehicle treated cells (Figure 4.8B). This was reduced to only 0.8-fold when NOFs were pre-treated with telmisartan before the addition of Ang II. all fold changes were plotted in relation to vehicle treated cells.

4.6 ADAM17 plays an important role in ET-1- and Ang II-stimulated paracrine signalling and HNSCC migration

The findings of this study indicate that ET-1 acts via both the ET_AR and ET_BR and Ang II via the AT_1R in NOFs. The signalling pathways by which they stimulate paracrine signalling between stromal NOFs and HNSCC cells was next investigated. It has previously been identified that ET-1 can stimulate the ADAM-mediated proteinase release of soluble, bioactive factors from the surface of cells (Murphy, 2008); the ability of a broad range MMP/ADAM inhibitor, GM6001, to

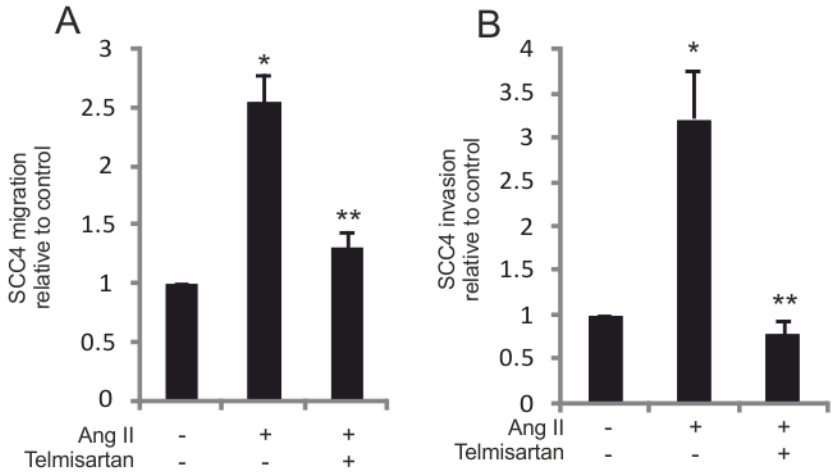


Figure 4.8 Ang II mediates paracrine stimulation of HNSCC migration and invasion via AT_1R: Ang II exerts most of its pathophysiological effects by binding to the AT_1R. To determine if Ang II exerts its paracrine effects via this receptor NOFs were pre-treated with a specific receptor antagonist to AT_1R, telmisartan (1 μM) for 30 min before the addition of Ang II (100 nM) for a further 4 h. Conditioned media was aspirated, filtered and added to the bottom Transwell migration well **(A)** or the bottom Matrigel invasion well **(B)**. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration or Matrigel invasion inserts in DMEM supplemented with 0.1% BSA. After 16 h (for migration) or 40 h (for invasion), cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p<0.05$ relative to Ang II treated cells (**).

influence the responses to ET-1 was next examined. NOFs were pre-treated with GM6001 for 30 min before the addition of ET-1. The conditioned media was aspirated from the NOFs, filtered and added to the bottom of the Transwell migration well. The application of the inhibitor blocked the ET-1 dependent paracrine stimulation of serum starved SCC4 cells that were left untreated before their addition to the Transwell insert. The presence of the inhibitor reduced SCC4 migration from 2.2-fold when NOFs were treated with ET-1 only, compared to untreated cells, to 1.0-fold (Figure 4.9A). This finding suggests that the soluble release of factors from the surface of NOFs and the involvement of a MMP or ADAMs enzyme, stimulated by treatment of the cells with ET-1, is a critical component in the paracrine signalling mechanism via which ET-1 promotes HNSCC cell migration and invasion.

As ADAMs have also been implicated in mediating the effects of Ang II in a number of contexts (Murphy, 2008), the same experiment was conducted for Ang II treatment. The treatment of NOFs with Ang II resulted in a 2.3-fold increase in SCC4 migration in comparison to vehicle treated cells (Figure 4.9B). A reduction in SCC4 migration was also observed when NOFs were pre-treated with GM6001 before Ang II treatment. The presence of the GM6001 reduced SCC4 migration to only 0.8-fold in comparison to vehicle treated cells. This finding implies that Ang II is also working via a similar signalling mechanism to that used and triggered by ET-1 stimulation.

The underlying signalling mechanism was further investigated by using siRNA to knock down the expression of the most common ADAMs proteinase implicated in the release of soluble factors from the surface of cells; ADAM17 (Murphy, 2008). NOFs were transiently transfected using siRNA to ADAM17 or siRNA to an insect transcript not expressed in human cells for 24 h. A mock transfection with Oligofectamine alone was also carried out. Following transfection all NOFs were treated with ET-1 or vehicle control for 4 h. The conditioned media collected from mock transfected NOFs treated with ET-1, resulted in an increase in SCC4 migration of 3.5-fold (Figure 4.10A). This was in comparison to conditioned media collected from mock transfected NOFs treated with serum free media. NOFs that were transfected with a scramble control duplex and treated with ET-1 provoked a similar increase in SCC4 migration to that observed for mock control, 3.2-fold. The knockdown of ADAM17 using siRNA, resulted in a reduction in SCC4 migration to 1.7-fold compared to mock transfected NOFs treated with serum free media. The reduction observed in SCC4 cell migration suggests that ADAM17 is vital in mediating the release of bioactive soluble factors from the surface of NOFs allowing them to act as ligands for HNSCC cells, stimulating their migratory ability. qPCR and immunoblot analysis were performed on RNA and protein lysate collected from NOFs transfected with siRNA as described previously to determine the extent of ADAM17 knockdown after

transfection. The transfection of NOFs with siRNA to ADAM17 resulted in a knockdown in transcript (Figure 4.10B) and protein (Figure 4.10C) levels by >50%; no significant effect was observed on the transcript levels of other ADAMs (-9, -10 and -15) expressed by the NOFs suggesting that ADAM17 is the only proteinase being targeted by the siRNA (Figure 4.10D). This mechanism was further investigated by the ADAM17 knockdown in NOFs 24 h prior to treatment with Ang II. The conditioned media collected from siRNA targeting an insect transcript transfected NOFs treated with Ang II, resulted in an increase in SCC4 migration 1.9–fold in comparison to NOFs transfected in the same way and treated with serum free media (Figure 4.11A). The knockdown of ADAM17 using siRNA, followed by treatment with Ang II resulted in a reduction in SCC4 migration to 0.7-fold, compared to control transfected NOFs treated with serum free media. The transfection of NOFs with siRNA to ADAM17 again abrogated the paracrine stimulation of Ang II on SCC4 cell migration. qPCR analysis was performed on pelleted NOFs to determine the extent of ADAM17 knockdown after transfection. The transfection of NOFs with siRNA to ADAM17 resulted in a knockdown in transcript levels by 50% (Figure 4.11B).

4.7 The ET-1 and Ang II stimulated paracrine signalling mechanism involves the transactivation of EGFR

It is apparent that the release of soluble factors, in part via ADAM17 proteinase activity, from the surface of NOFs is crucial in the paracrine stimulation of HNSCC migration and invasion. Epidermal growth factor receptor (EGFR) signalling is a pathway commonly deregulated in a number of cancers. The pathway can be activated via the binding of soluble factors released from the cell via an autocrine manner, from other surrounding cells via a paracrine manner and via intracellular signalling mechanisms; ADAM17 has been implicated in this process (Blobel, 2005). These soluble factors can act as ligands for the EGFR. Aberrant activation of the EGFR can promote an increase in cell proliferation, migration and invasion and is a common feature of many cancer types. It was therefore decided to examine the involvement of EGFR signalling in the actions of ET-1 and Ang II in HNSCC. SCC4 cells were pre-treated with an antagonist of EGFR, AG 1478, for 30 min before the addition of the cells to the top of the Transwell migration well. The presence of AG 1478 reduced the effect of conditioned media collected from NOFs pre-treated with ET-1 or Ang II on SCC4 migration. SCC4 cell migration in the presence of ET-1 stimulated conditioned media was reduced from 3.0-fold to 1.2-fold (compared to vehicle only) when the SCC4 cells were pre-treated with the AG 1478 antagonist (Figure 4.12A). SCC4 cell migration in the presence of Ang II stimulated conditioned media was reduced from 2.2-fold to 0.6-fold (compared to vehicle only) when the SCC4 cells were pre-treated with the AG 1478 antagonist (Figure 4.12B). This suggests that soluble ectodomains

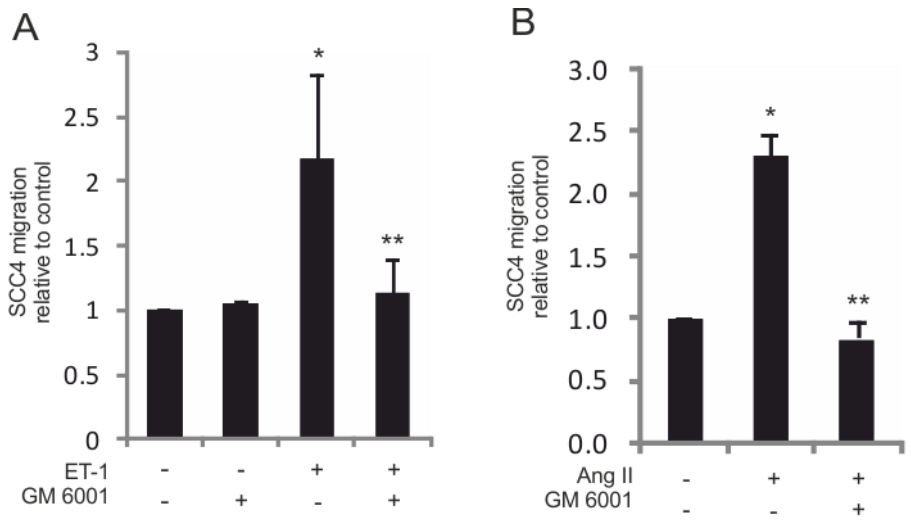


Figure 4.9 The release of a soluble factor from the surface of NOFs is a critical step in ET-1 and Ang II mediated HNSCC cell migration: The signalling pathways in which ET-1 and Ang II stimulate paracrine signalling between NOFs and HNSCC cells was investigated. ET-1 is known to stimulate the ADAM-mediated proteinase release of soluble, bioactive factors from the surface of cells. NOFs were pre-treated with an ADAM inhibitor, GM6001 (10 µM) for 30 min before the addition of ET-1 (10 nM) **(A)** or Ang II (100 nM) **(B)** for a further 4 h. Conditioned media was aspirated, filtered and added to the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p<0.05$ relative to ET-1 or Ang II treated cells (**).

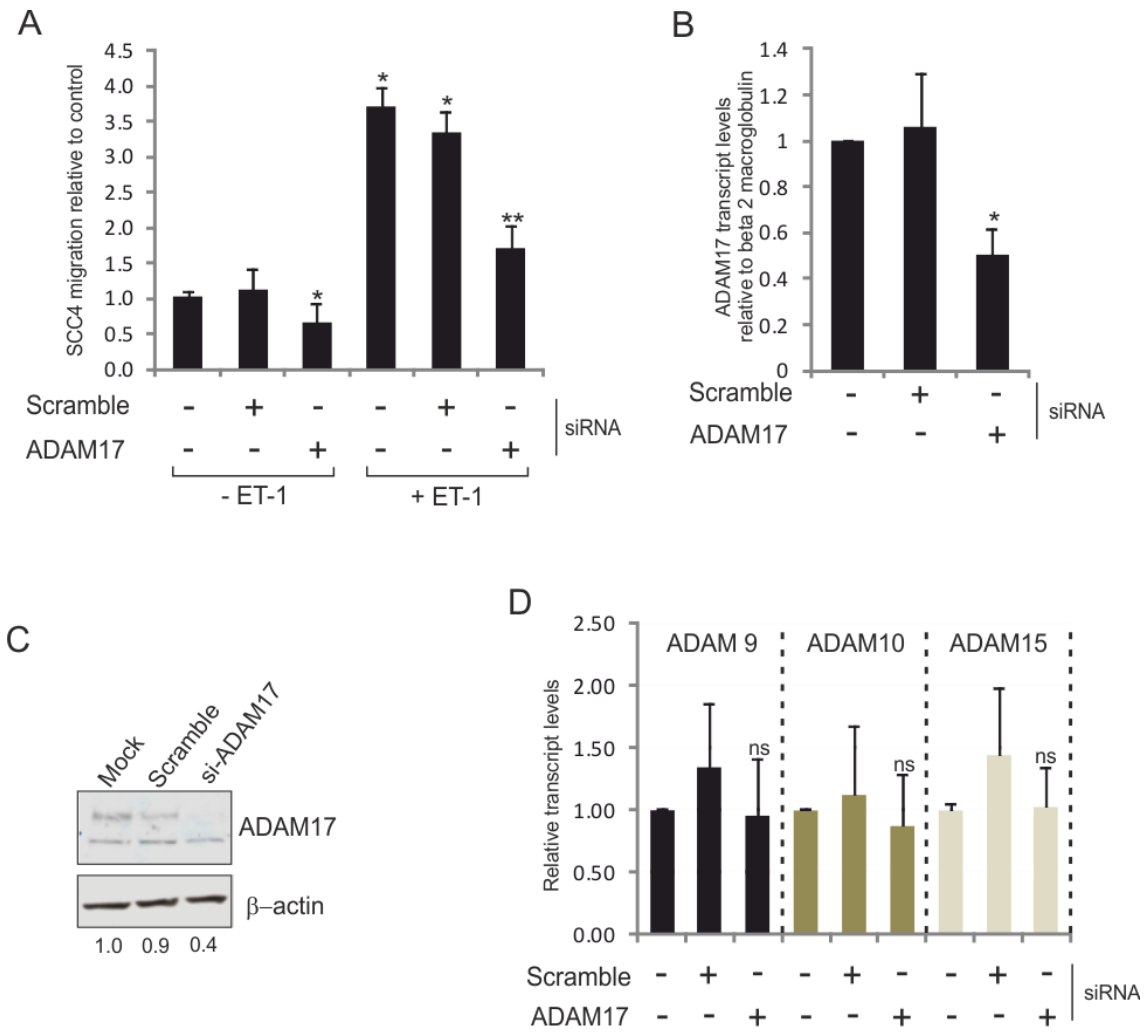


Figure 4.10 ADAM17 plays an important role in ET-1-stimulated paracrine signalling and HNSCC migration: ADAM17 is a member of the ADAMs proteinase family and is responsible for the release of soluble factors from the surface of cells. NOFs were transiently transfected with siRNA to ADAM17 for 24 h. The cells were treated with ET-1 (10 nM) for 4 h. Conditioned media was aspirated, filtered and added to the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope **(A)**. Transfected NOFs were harvested and RNA extracted or lysates prepared. qPCR analysis was used to measure the transcript levels of ADAM17 **(B)**; to determine transfection efficiency, other ADAMs proteinases (ADAM9, 10 and 15, as indicated); to indicate the specificity of siRNA for ADAM17 **(D)** or U6 as a reference gene. Cell lysates were separated by SDS-PAGE and immunoblotted for ADAM17 and β-actin (as a loading control). A representative blot is shown **(C)** and the intensity of the band corresponding to ADAM17, determined by densitometry and normalized to β-actin levels in the same sample, is indicated under each lane. Each data point represents an average of at least 3 independent experiments, carried out in triplicate. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*) relative to untreated control, ns = not significant.

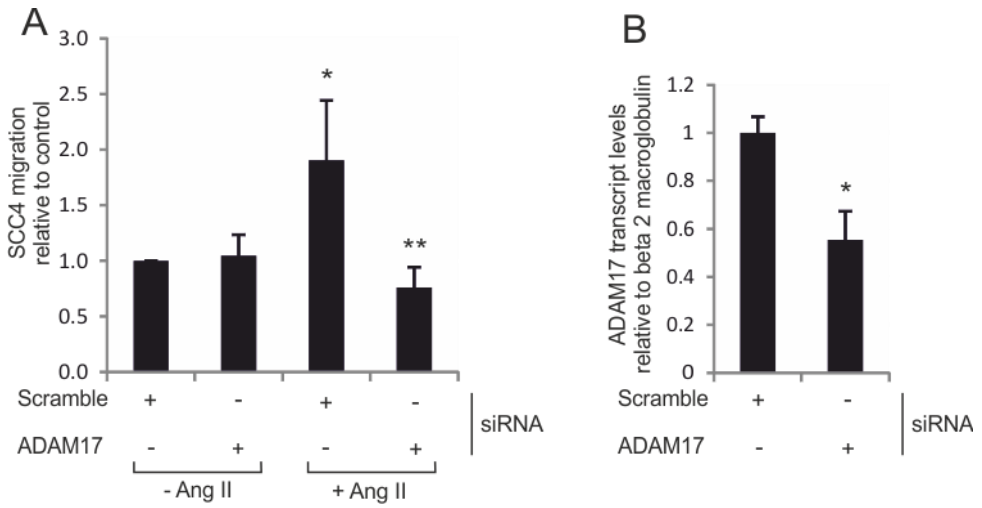


Figure 4.11 ADAM17 plays an important role in Ang II-stimulated paracrine signalling and HNSCC migration: ADAM17 is a member of the ADAMs proteinase family and is responsible for the release of soluble factors from the surface of cells. NOFs were transiently transfected with siRNA to ADAM17 for 24 h. The cells were treated with Ang II (100 nM) for 4 h. Conditioned media was aspirated, filtered and added to the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope **(A)**. Transfected NOFs were harvested and RNA extracted or lysates prepared. qPCR analysis was used to measure the transcript levels of ADAM17 **(B)**. Each data point represents an average of at least 3 independent experiments, carried out in triplicate. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*) relative to untreated control, ns = not significant.

released from the surface of the fibroblast cells via ADAM17 proteolysis, can act as ligands for EGFR resulting in its activation and triggering downstream signalling which results in the increased migratory ability of the HNSCC cells. The observation that the receptor antagonist to the EGFR resulted in a decrease in paracrine stimulation of SCC4 cells suggested the following experiment in which the inhibition of not only the EGFR but the combined effect of inhibition with ET_AR and ET_BR was investigated. SCC4 cells were pre-treated with AG 1478 before their addition to the Transwell migration assay and NOFs were pre-treated with a specific ET_AR antagonist, BQ-123 and a specific ET_BR antagonist, BQ-788 for 30 min before their stimulation with ET-1. The combined inhibition of both the ET_AR and ET_BR and the EGFR resulted in further reduction in migration in response to ET-1. ET-1 treatment alone resulted in the usual increase in SCC4 migration. In this experiment migration was increased 4.0-fold in comparison to vehicle treated cells (Figure 4.13A). When SCC4 cells were pre-treated with AG 1478 before their addition to the migration assay their migration was reduced to only 2.0-fold. The pre-treatment of NOFs with BQ-123 reduced migration to only 2.2-fold and pre-treatment with BQ-788 reduced migration to 2.0-fold. The presence of AG 1478 significantly reduced this further to 1.3- and 1.5-fold for BQ-123 and BQ-788, respectively. The pre-treatment of NOFs with both the BQ-123 and the BQ-788 receptor antagonists further reduced the paracrine stimulation of SCC4 cellular migration to only 1.5-fold in comparison to treatment of the NOFs with the individual antagonists. When AG 1478 was added this again significantly reduced to migration to only 1.0-fold. All treatments were plotted relative to untreated NOFs and SCC4 cells.

To further investigate the suggestion that soluble factors released from the surface of NOFs can potentially act as ligands for the EGFR, conditioned media collected from ET-1 treated NOFs was incubated for 30 min with neutralising antibodies to three known EGFR ligands; tumour growth factor-α (TGF-α), heparin bound-EGF (HB-EGF) and amphiregulin, all of which have been implicated in the progression of a number of malignancies. The incubation of the individual neutralising antibodies with conditioned media blocked stimulation of SCC4 migration, further supporting the implication that EGFR transactivation is important in the paracrine stimulation of HNSCC cells (Figure 4.14A). This inhibition of cell migration was further potentiated when the conditioned media was incubated with all three neutralising antibodies before its addition to the Transwell migration assay. There was no change in SCC4 migration when all three neutralising antibodies were added alone and in combination to media collected from vehicle treated NOFs. When NOFs were treated with ET-1 and the conditioned media collected and added directly to the Transwell migration well without further incubation with the neutralising antibodies, migration was increased 3.0-fold in

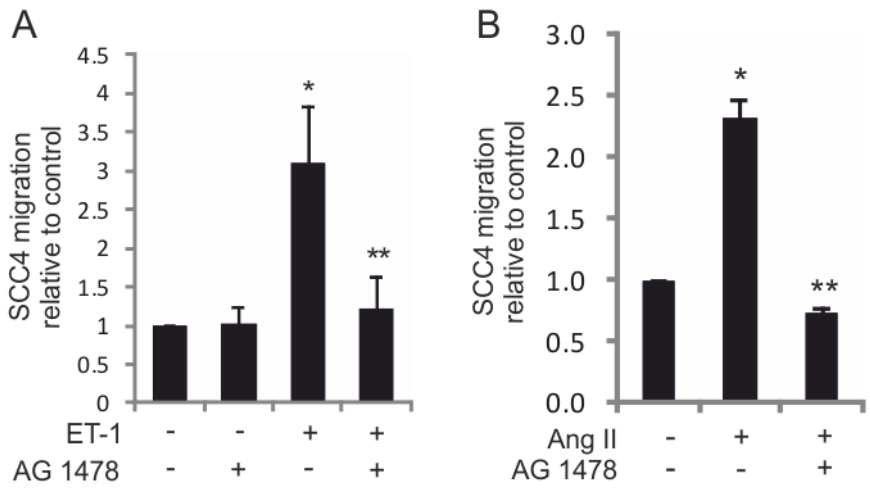


Figure 4.12 EGFR transactivation is involved in the paracrine stimulation of HNSCC migration by ET-1 and Ang II: EGFR signalling is a pathway commonly associated with deregulation in a number of cancers. EGFR signalling is triggered by autocrine and paracrine stimulation. Soluble factors released from cells can act as ligands for the EGFR, resulting in its dimerisation. Conditioned media collected from NOFs treated with 10 nM ET-1 **(A)** or 100 nM Ang II **(B)** for 4 h was added to the bottom Transwell migration well. SCC4 cells were pre-treated with an EGFR antagonist, AG 1478 (125 nM) for 30 min. $1x10^5$ serum starved cells treated as above were added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA with DMEM. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p<0.05$ relative to ET-1 or Ang II treated cells (**).

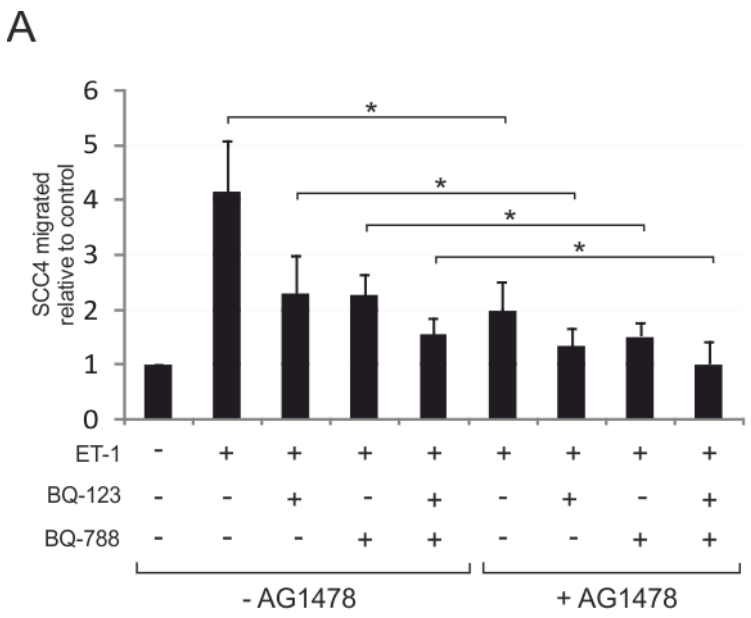


Figure 4.13 Combining treatment with antagonists to ETAR and ETBR and an inhibitor to EGFR further increase the inhibition of ET-1 stimulated HNSCC migration: NOFs were pre-treated with a specific receptor antagonist to ETAR, BQ-123 (1 μM) or to ETBR, BQ-788 (1 μM) for 30 min before the addition ET-1 (10 nM) for a further 4 h. Conditioned media was aspirated, filtered and added to the bottom Transwell migration well. SCC4 cells were pre-treated with an EGFR antagonist, AG 1478 (125 nM) for 30 min before their addition to the top of a Transwell migration insert **(A)**. $1x10^5$ serum starved SCC4 cells treated as above were added to the top of the Transwell migration or Matrigel invasion inserts in DMEM supplemented with 0.1% (w/v) BSA. After 16 h cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control, or as indicated by bars and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*).

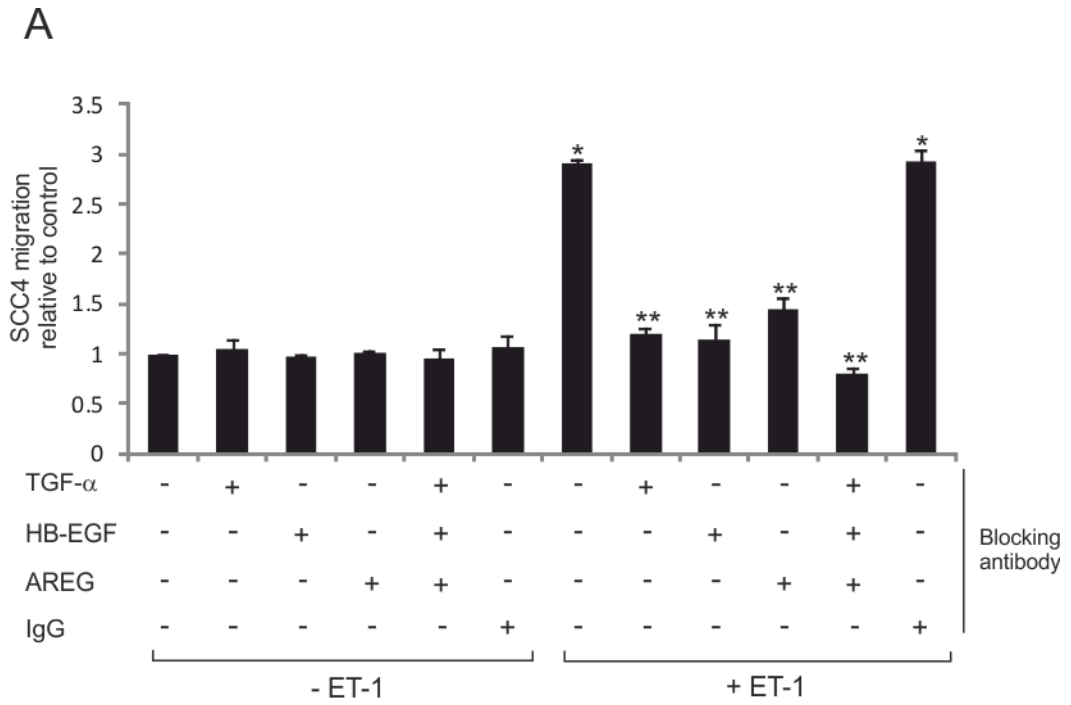


Figure 4.14 Neutralising antibodies to EGFR ligands can block ET-1 stimulated HNSCC migration: The effect of neutralising antibodies on ET-1 paracrine stimulated SCC4 migration was investigated. NOFs were treated with ET-1 (10 nM) for 4 h before the media was aspirated and filtered. The conditioned media collected was incubated alone and in combination with neutralising antibodies to three EGFR ligands; TGF-α, HB-EGF and amphiregulin (10 μl/ml). The media and antibodies were incubated together on a blood wheel for 30 min before being added to the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% BSA (w/v) **(A)**. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p<0.05$ relative to ET-1 treated cells (**).

comparison to vehicle treated NOFs. SCC4 migration was reduced when conditioned media was incubated with a neutralising antibody to TGF-α to 1.3-fold, 1.2-fold with a neutralising antibody to HB-EGF, 1.5-fold with a neutralising antibody to amphiregulin and when all three were combined migration was reduced to 0.8-fold. Incubation of conditioned media with an isotype control antibody had no effect on SCC4 migration. All results were plotted relative to untreated cells.

4.8 EGFR transactivation by ET-1 induces COX-2 expression

The results of this study have shown that ET-1 promotes HNSCC cell migration in the presence of NOFs via a paracrine mechanism. The ability of the mitogenic peptide to regulate genes known to be altered by EGFR signalling during this process was next investigated. BK, another mitogenic peptide, is known to cause an increase in cyclooxygenase-2 (COX-2) expression in HNSCC cells via the phosphorylation and activation of the EGFR and the mitogen-activated protein kinase (MAPK) pathway (Zhang *et al*, 2008). Elevated expression of COX-2 is known to be associated with an invasive phenotype and a poor prognosis in HNSCC (Chan *et al*, 1999). COX-2 is responsible for the conversion of arachidonic acid into prostaglandin E_2 (PGE_2) which can promote increased proliferation, cell survival, angiogenesis, migration and invasion of tumour cells.

In order to assess any involvement of COX-2 in the observed effects of ET-1, SCC4 cells were seeded into 12-well titre plates and were allowed to adhere overnight. The following day cells were serum starved for 24 h. SCC4 cells were pre-treated with the EGFR antagonist, AG 1478 or a vehicle control for 30 min before being treated directly with conditioned media that was prepared from NOFs treated with ET-1 or vehicle control for 4 h. Cells were pelleted 4 h after treatment and subjected to qPCR analysis. qPCR analysis of mRNA isolated from SCC4 cells pre-treated with vehicle control and then treated with conditioned media collected from NOFs treated with ET-1, showed that ET-1 increased the expression of COX-2 1.7-fold compared to untreated SCC4 cells (Figure 4.15A). The pre-treatment of SCC4 cells with AG 1478 before treatment with conditioned media collected from NOFs treated with ET-1, blocked the increase in COX-2 expression. Treatment of SCC4 with AG 1478 resulted in a decrease in COX-2 transcript levels (0.5-fold in comparison to vehicle treated cells), suggesting that EGFR signalling may regulate basal COX-2 expression as well as that induced by ET-1. To investigate if COX-2 directly contributed to promoting the migration of HNSCC cells in this system, cells were pre-treated with the COX inhibitor ibuprofen; which inhibits both COX-1 and COX-2, or the COX-2 specific inhibitor celecoxib for 30 min before their addition to the migration insert. The presence of both inhibitors blocked the induction of SCC4 cell migration to 0.3-fold and 1.0-fold, respectively, invoked by conditioned media collected from ET-1 stimulated NOFs

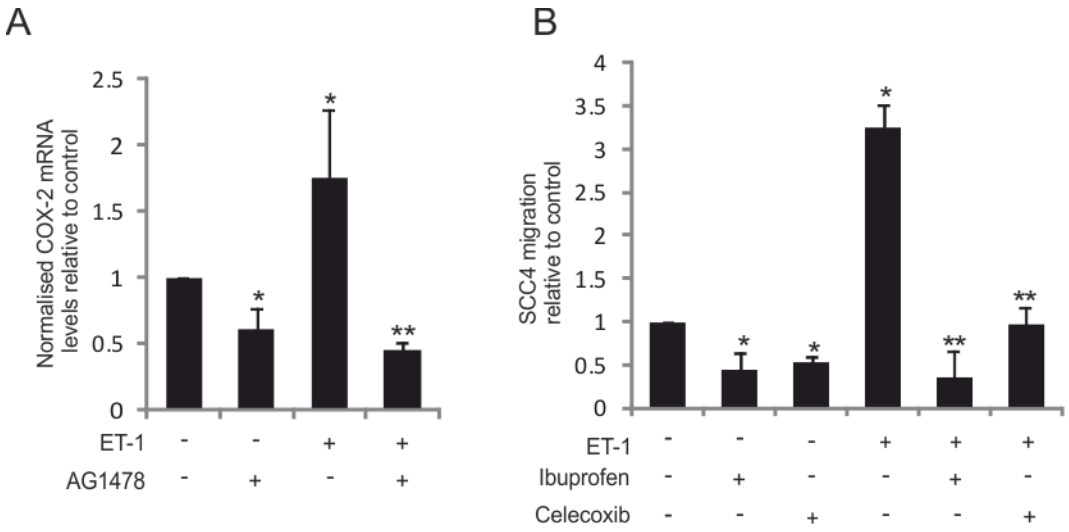


Figure 4.15 Paracrine transactivation of EGFR by ET-1 stimulates COX-2 expression and function: In order to investigate the change in COX-2 gene expression in response to conditioned media treatment SCC4 cells were seeded and serum starved before being pre-treated with an EGFR antagonist, AG 1478 (125 nM) for 30 min **(A)**. NOFs were treated with a ET-1 (10 nM) for 4 h. Conditioned media was aspirated, filtered and either added directly to the SCC4 cells or added to the bottom Transwell migration well. Seeded SCC4 cells were treated for 4 h before being pelleted and RNA extracted and subject to qPCR analysis for COX-2 or U6 as a reference gene. SCC4 cells were pre-treated with a COX inhibitor, ibuprofen (100 µM) or a COX-2 inhibitor, celecoxib (50 µM) for 30 min **(B)**. $1x10^5$ serum starved SCC4 cells treated as above were added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% BSA (w/v). After 16 h cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control, or as indicated by bars and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p<0.05$ relative to ET-1 treated cells (**).

(Figure 4.14B). Untreated SCC4 cells migrated 3.2-fold more in the presence of ET-1 stimulated conditioned media than in unstimulated conditioned media. The fold change was plotted relative to vehicle treated cells. The inhibitors also reduced SCC4 migration when conditioned media was collected from NOFs treated with vehicle control. Ibuprofen reduced migration to 0.4-fold and celecoxib reduced it to 0.5-fold, both in comparison to vehicle treated SCC4 cells and NOFs. These findings corroborate previous suggestions of a critical role for COX-2 in stromal-epithelial interactions in HNSCC (Thomas *et al*, 2006).

4.9 miR-145 reduces the ability of NOFs to promote SCC4 migration

It has recently been shown that microRNAs (miRNAs) regulate the expression of different genes that play important roles in cancer cell invasion, migration and metastasis (Nicoloso *et al*, 2009). miRNAs are short, naturally occurring non-coding RNAs which regulate the expression of target genes. The mature miRNA is single stranded and made up of around 21 to 25 nucleotides (Kim, 2005). The production of miRNAs is executed in a well-coordinated manner. miRNAs begin their life as a long section of primary transcript, around several kilobases in length called the pri-miRNA. This is produced in a polymerase II-dependent manner. The transcript contains a hairpin structure, a 5' CAP structure and a 3' polyadenylated tail. The stem-loop structure is cleaved by the nuclear RNase III known as Drosha and its double stranded binding protein DGCR8, to produce pre-miRNA. The remaining regions are thought to be degraded in the nucleus. This maturational process of miRNAs is stimulated and regulated in response to proliferative stimuli and cellular differentiation. Once the pre-miRNA is produced it is transported out of the nucleus into the cytoplasm. This transportation process is triggered and executed by exportin-5 and its cofactor Ran-GTP. Exportin-5 is a nuclear export factor and is also thought to stabilise pre-miRNA. The export factor targets its cargo and transports it into the cytoplasm through a nuclear pore. In the cytoplasm a RNase III nuclease called Dicer processes the pre-miRNA into a duplex of around 22 nucleotides in length via a process known as dicing. The miRNA duplex does not remain in this form for long. It is incorporated into a RNA induced silencing complex (RISC) that also contains argonaute. After the production of this complex only one strand is selected from it to become the mature miRNA and will therefore remain in the mature RISC. This strand is known as the guide strand. The other remaining strand is degraded. The decision as to which strand is chosen and which strand is discarded is thought to be based on the stability of duplex in the 5' half (Kim, 2005). miRNAs regulate gene expression at a post-transcriptional level through the selective silencing of target messenger RNAs (mRNAs) (Medina and Slack, 2008). They achieve this by binding to specific sequences in the 3' untranslated region (3'UTR) of their

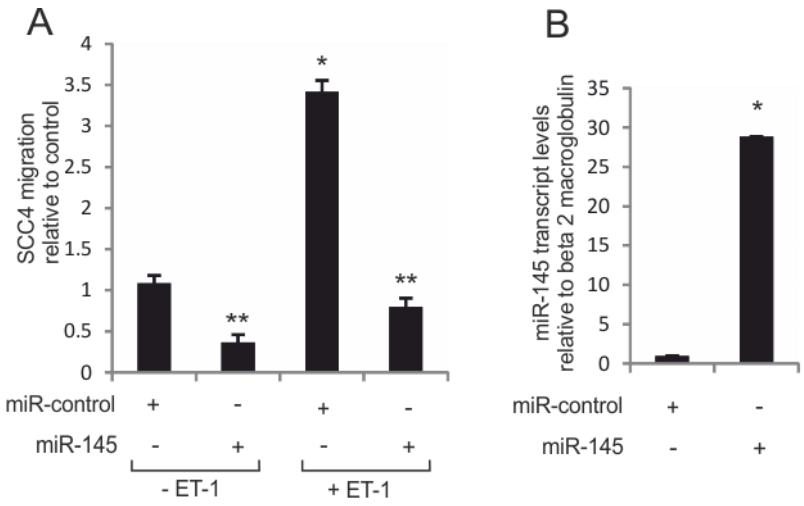


Figure 4.16 miR-145 can inhibit ET-1-stimulated paracrine HNSCC migration: NOFs were transiently transfected with miR-145 for 24 h. The cells were treated with ET-1 (10 nM) for 4 h. Conditioned media was aspirated, filtered and added to the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope **(A)**. Transfected NOFs were harvested and RNA extracted or lysates prepared. qPCR analysis was used to measure the transcript levels of miR-145 **(B)**. Each data point represents an average of at least 3 independent experiments, carried out in triplicate. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*) relative to untreated control, $p<0.05$ relative to miR-control transfection +ET-1 treatment (**).

target gene's transcripts (Zeng and Cullen, 2005). Computer analysis studies have suggested that the miRNAs could target up to 10% of all human genes (John *et al*, 2004).

It is known that miRNAs can act as tumour suppressors (Calin and Croce, 2006) and miR-145 has been shown to be down regulated in a number of malignancies (Sachdeva *et al*, 2009). Work conducted previously in the lab has shown that miR-145, a putative tumour-suppressor (unpublished data generated in the laboratory), is down-regulated in oral cancer and can repress migration of cancer cells in response to mitogenic peptides, by down regulating ADAM17. The work presented in this project has identified the involvement of ADAM17 in mitogenic peptide-induced stromal epithelial cross-talk; it was therefore decided to examine the effect of modulating miR-145 on this pathway. qPCR data has identified the presence of mature miR-145 in NOFs (unpublished data generated in the laboratory). The effect that over expressing miR-145 in NOFs has on the migration of SCC4 cells in the presence and absence of ET-1 and Ang II was investigated. NOFs were transiently transfected with miR-145 or miR-control for 24 h before treatment with ET-1, Ang II or serum free media for a further 4 h. After this time period the media was aspirated, filtered and added to the bottom of the Transwell migration assay. Serum starved SCC4 cells were left untreated and were added to the top chamber of the assay. The cells were allowed to migrate for 16 h before they were fixed, stained and counted as described in Section 2.2.4. Cells transfected with miR-control followed by ET-1 treatment resulted in an increase in SCC4 migration of 3.4-fold (Figure 4.16A). This stimulation of migration was significantly reduced to 0.9-fold when NOFs were transfected with miR-145 before treatment with ET-1. Transfection of NOFs with miR-145 followed by treatment for 4 h with serum free media also resulted in a small but significant decrease of 0.4-fold in SCC4 migration in comparison to NOFs transfected with miR-control and treated with serum free media. qPCR analysis was conducted on transfected NOFs to confirm the over expression of miR-145. Transient transfection of the cells with miR-145 resulted in an increase of 29-fold in the expression of the mature miRNA (Figure 4.16B).

NOFs transfected with miR-control and treated with Ang II resulted in an increase in migration of SCC4 cells of 1.9-fold in comparison to treatment with serum free media of NOFs also transfected with miR-control (Figure 4.17A). When NOFs were transfected with miR-145 and again treated with Ang II this resulted in a decrease in SCC4 migration to 0.6-fold. NOFs that were transfected with miR-control and treated with serum free media again resulted in a small decrease in SCC4 migration of 0.7-fold similar to that observed in the ET-1 set of experiments. However this reduction in SCC4 migration was not significant. qPCR analysis was conducted on transfected NOFs to confirm the over expression of miR-145. Transient transfection of the

cells with miR-145 resulted in an increase of 25-fold in the expression of the mature miRNA (Figure 4.17B).

miR-145 is known to target ADAM17 and could therefore be restricting the ET-1 and Ang II stimulated release of bioactive ligands which trigger migration via binding to the EGFR on HNSCC cells. This level of inhibition is greater than that observed with siRNA knockdown of ADAM17 in NOFs, suggesting the involvement of other miR-145 targets.

4.10 The over expression of ECE-1c in NOFs potentiates the paracrine stimulation of HNSCC migration

ECE-1c is one of the four ECE-1 isoforms. It is the most abundantly expressed and is located at the cell surface (Muller *et al*, 2003). The expression of ECE-1c is known to be elevated in a number of cancers (Muller *et al*, 2003). This project has identified that ECE-1 is also over expressed in cell lines isolated from primary HNSCCs, oral dysplasias and a local metastasis and NOFs in comparison to NOKs (Figure 3.6B and Figure 3.8A). This study has shown that heterologous over-expression of ECE-1c in SCC4 cells stimulated an increase in their migration 1.4–fold in comparison to cells transfected with a control plasmid (Figure 3.9A). This result and that concluding that ECE-1 is over expressed in NOFs in comparison to NOKs prompted the effect of the over expression of ECE-1c in NOFs on the paracrine stimulation of SCC4 cell migration to be investigated. NOFs were transiently transfected using FuGENE 6 with a pcDNA3 vector containing the ECE-1c coding region or a control pcDNA3 vector for 24 h. After 24 h the cells were treated for a further 4 h with serum free media. After the allocated time period the media was aspirated, filtered and added to the bottom well of Transwell migration assay. SCC4 were left untreated and added to the top of the Transwell migration assay. The cells were allowed to migrate through the permeable membrane for 16 h before they were fixed, stained and counted using the method described in Section 2.2.4. Over expression of ECE-1c in NOFs, resulted in an increase in SCC4 migration of 3.4-fold in comparison to NOFs transfected with the plasmid control (Figure 4.18A). qPCR analysis was conducted on NOFs to determine the level of expression of ECE-1. The expression of ECE-1 increased dramatically (50-fold) in NOFs after transfection with the ECE-1c isoform in comparison to the level normally expressed within the cells (Figure 4.18B).

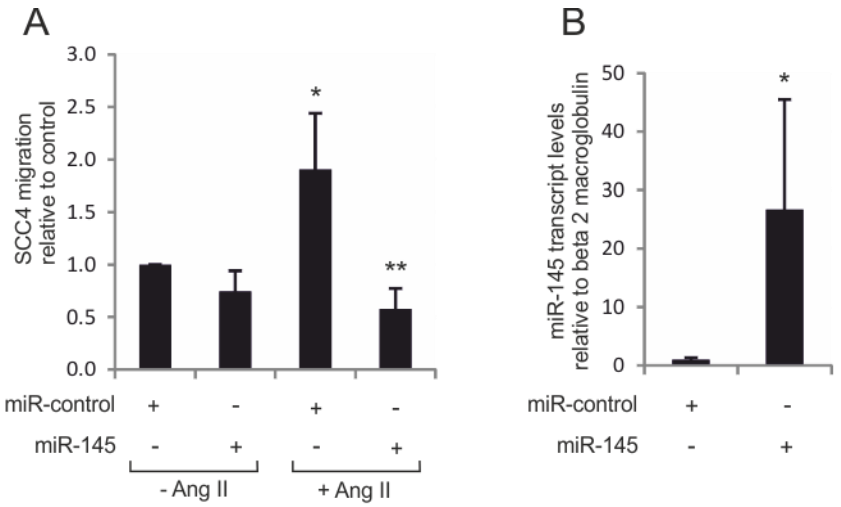


Figure 4.17 miR-145 can inhibit Ang II-stimulated paracrine HNSCC migration: NOFs were transiently transfected with miR-145 for 24 h. The cells were treated with Ang II (100 nM) for 4 h. Conditioned media was aspirated, filtered and added to the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope **(A)**. Transfected NOFs were harvested and RNA extracted or lysates prepared. qPCR analysis was used to measure the transcript levels of miR-145 **(B)**. Each data point represents an average of at least 3 independent experiments, carried out in triplicate. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*) relative to untreated control, $p<0.05$ relative to miR-control transfection +Ang II treatment (**).

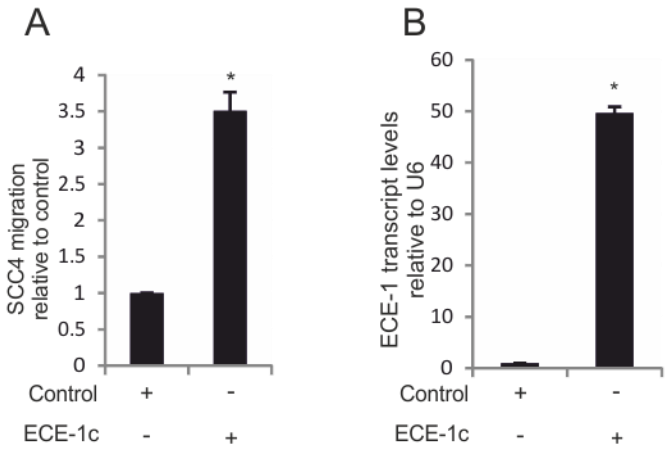


Figure 4.18 Over expression of ECE-1c in NOFs promotes migration of SCC4 cells: NOFs were transiently transfected with a pcDNA3 vector contain the ECE-1c coding region for 24 h before the addition of serum free media for 4 h. The conditioned media was aspirated, filtered and added to the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope **(A)**. Transfected NOFs were harvested and RNA extracted or lysates prepared. qPCR analysis was used to measure the transcript levels of ECE-1 **(B)**. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p<0.05$ relative to control transfected cells.

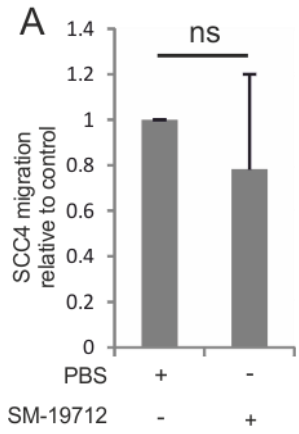


Figure 4.19 ECE-1 inhibition does not block pacracrine stimulated SCC4 migration: ECE-1 is known to cleave biologically active ET-1 from big ET-1. NOFs cells were treated with an ECE-1 inhibitor, SM-19712 (10 µM) **(A)**. The conditioned media was aspirated, filtered and added to the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), ns = not significant.

4.11 Inhibition of ECE-1 activity in NOFs does not reduce paracrine-stimulated HNSCC migration

Having shown that over-expression of ECE-1c in NOF is able to modulate the behaviour of HNSCC cells, the effect of inhibiting ECE-1 activity in NOFs on SCC4 migration was investigated. NOFs were treated with a broad range ECE-1 inhibitor, SM-1972 for 4 h before the media was aspirated, filtered and added to the bottom well of the Transwell migration assay. Untreated SCC4 cells were added to the assay and allowed to migrate for 16 h (as described in Section 2.2.4). Treatment of NOFs with the SM-1972 inhibitor resulted in a decrease in SCC4 migration 0.8-fold in comparison to untreated NOFs (Figure 4.19A). This reduction in SCC4 migration however was not significant.

4.12 The over expression of ACE in NOFs potentiates the paracrine stimulation of HNSCC migration

Evidence has been provided in this thesis that the over expression of ACE in SCC4 cells stimulated an increase in their migration 1.5–fold in comparison to cells transfected with a pcDNA3 control plasmid (Figure 3.5B). The effect of over expression of ACE in NOFs on the paracrine stimulation of SCC4 cell migration was investigated. NOFs were transiently transfected using FuGENE 6 with a pIRES vector containing the coding region of somatic ACE or a control pcDNA3 vector for 24 h. After 24 h NOFs were treated for a further 4 h with serum free media. The conditioned media was added to the Tanswell migration assay and the ability of SCC4 cells to migrate was investigated (as previously described in Section 2.2.4). Over expression of ACE in NOFs, resulted in an increase in SCC4 migration of 2.7-fold in comparison to NOFs transfected with a control vector (Figure 4.20A). qPCR analysis was conducted on NOFs to determine the level of expression of ACE. The expression of ACE increased 36-fold in NOFs after transfection with somatic ACE in comparison to the level normally expressed within the cells (Figure 4.20B).

4.13 Summary

The invasion and migration of cancer cells is increasingly recognised to be influenced by factors derived from adjacent tumour-associated stroma. The contextual signals regulating these stromal-tumour interactions, however, remain poorly understood. Within this chapter a role for the mitogenic peptides ET-1 and Ang II, in promoting pro-metastatic cross-talk between HNSCC cells and adjacent NOFs was investigated.

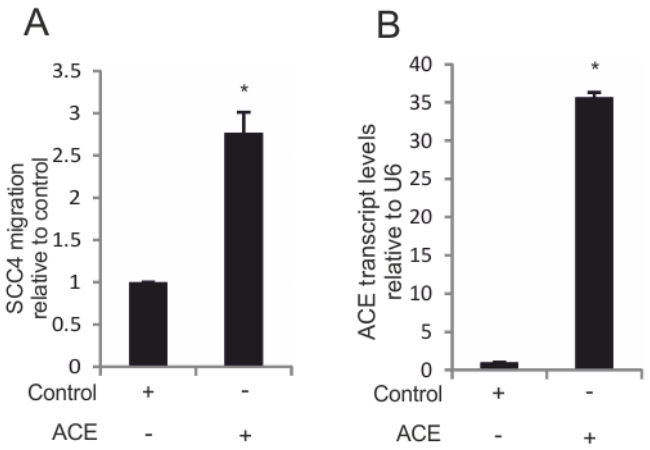


Figure 4.20 Over expression of ACE in NOFs promotes migration of SCC4: NOFs were transiently transfected with a pIRES vector containing somatic ACE for 24 h before the addition of serum free media for 4 h. The conditioned media was aspirated, filtered and added to the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope **(A)**. Transfected NOFs were harvested and RNA extracted or lysates prepared. qPCR analysis was used to measure the transcript levels of ECE-1c **(B)**. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p<0.05$ relative to control transfected cells.

Initial studies within the chapter identified that ET-1 can exert its effects through both the ET_AR and the ET_BR. Ang II binds to and exerts its effects through the AT_1R. The treatment of NOFs with ET-1 and Ang II activates ADAM17 mediated release of EGFR ligands including TGF-α, HB-EGF and amphiregulin which trigger EGFR signalling resulting in an increase in the migration and invasion of HNSCC cells. ET-1 mediated paracrine transactivation of EGFR also increased COX-2 levels in the HNSCC cells, providing a molecular insight into the mechanisms by which the elevated levels of ET-1 observed in head and neck cancers may contribute to disease progression.

This chapter also identifies that the miRNA, miR-145, is an important regulator of paracrine stimulation of HNSCC migration triggered by treatment of NOFs with ET-1 and Ang II. miR-145 can repress the activation of ADAM17 which as mentioned before is crucial in the ability of the mitogenic peptides to exert their effects on the promotion of migration of the HNSCC cells.

Final experiments conducted within this chapter begin to look at the role of the enzymes responsible for the production of biologically active ET-1 and Ang II. The over expression of ECE-1c in NOFs, resulted in the paracrine stimulation of HNSCC migration. This result is similar to that observed for the over expression of ACE within the same cell type. These findings suggest that the enzymes could be responsible for the production of both ET-1 and Ang II at a local level within the head and neck tumour microenvironment. The inhibition of ECE-1 however does not result in a decrease in the paracrine stimulation of HNSCC cells suggesting that other factors aside from the enzyme are responsible for the production of ET-1.

The findings within this chapter provide novel molecule detail regarding the role of the tumour microenvironment and the tumour-stromal interactions that occur within it in contributing to the progression of HNSCC. The chapter also identifies potential targets for new drug therapies in the treatment of the disease.

Chapter 5: The effect of ET-1 and Ang II on the phenotype of normal oral fibroblasts

5.1 Introduction

Tumour cells do not exist alone; the development and progression of epithelial tumours is profoundly influenced by the surrounding tumour microenvironment (the reactive stroma). The stroma is composed of fibroblasts, blood vessels, lymphatics, extracellular matrix components and immune cells (Stover *et al*, 2007). Factors released by the tumour cells, and other cells in the microenvironment, can bind to the surrounding fibroblasts, the most common cell type found within the stroma, and cause a change in their phenotype. Fibroblasts are non-vascular, non-epithelial and non-inflammatory cells found within connective tissue (Tarin and Croft, 1969). Fibroblasts play an important role in regulating the differentiation of epithelial cells, extra cellular matrix (ECM) deposition, inflammation and wound healing. Fibroblasts can become activated by factors present within the tumoural environment; assuming a myofibroblastic phenotype resembling that of fibroblasts found in healing wounds and fibrosis. The change in their activation state can result in alterations in the factors that they release. These factors in turn can bind to the nearby epithelial tumour cells promoting and producing stromal-tumour interactions. The importance of the tumour microenvironment in the progression of epithelial tumours is becoming increasingly recognized and suggestions have been made that it could present a possible new target for the development of therapeutic approaches for the treatment of cancer.

The previous data and experiments presented in this thesis have implicated normal oral fibroblasts (NOFs) in the paracrine signalling mechanism responsible for the increase in head and neck squamous cell carcinoma (HNSCC) cell migration and invasion. Endothelin-1 (ET-1) and angiotensin II (Ang II) are both capable of promoting paracrine signalling between NOFs and HNSCC cells via the stimulation and release of pro-invasive factors. It was therefore hypothesised that both ET-1 and Ang II stimulate paracrine signalling between NOFs and malignant epithelial cells by modifying the NOFs phenotype. The effect of both peptides on the NOFs phenotype including their ability to promote proliferation, migration and cellular contraction of the cells was therefore investigated.

5.2 ET-1 stimulates proliferation of NOFs

An increase in cellular proliferation is associated with an 'activated' fibroblast phenotype. The effect of ET-1 on the proliferation NOFs was investigated. NOFs were seeded into 96-well titre plates and allowed to reach 40% confluency. The cells were treated with ET-1 and allowed to proliferate for 48 h. The treatment of NOFs with ET-1 resulted in a small (10%) but significant ($p<0.05$) increase in their proliferation (Figure 5.1A). The pre-treatment of NOFs with a specific

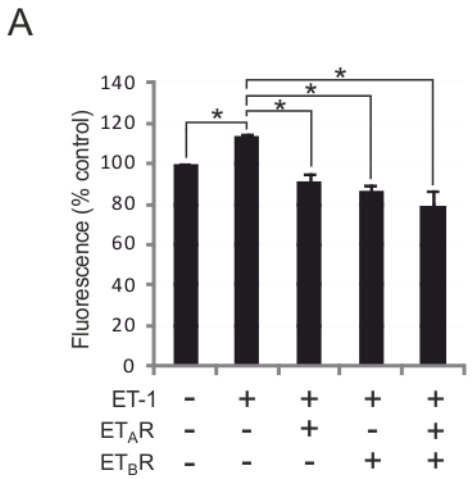


Figure 5.1 ET-1 stimulates NOF proliferation: Fibroblasts found within the stroma of the tumour microenvironment can undergo phenotypic changes including an increase in their proliferative ability. In order to determine the effect of ET-1 on the proliferation of NOFs, the cells were treated with ET-1 (10 nM) and assayed using a MTS proliferation assay **(A)**. Briefly, NOFs were cultured in 96 well titre plates and allowed to adhere overnight. The cells were pre-treated with a specific receptor antagonist to ET_AR, BQ-123 (1 μM) and/or a specific receptor antagonist to ET_BR, BQ-788 (1 μM) for 30 min before the addition of ET-1 (10 nM). 20 μL MTS reagent was added to each well at 0 h and 48 h time points and fluorescence was measured at 492 nm. Each data point represents an average of at least 3 independent experiments, carried out in triplicate. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*) relative to untreated control, or as indicated by bars.

ET_AR antagonist, BQ-123 and a specific ET_BR antagonist, BQ-788 both alone and in combination blocked the proliferation of the cells stimulated by ET-1 treatment. This again suggests that ET-1 is exerting its effect through both receptors. Treatment of NOFs with the ET_BR antagonist resulted in a greater reduction in NOF proliferation in comparison to treatment with the receptor antagonist to ET_AR (25% vs. 20% in comparison to ET-1 treated NOFs, respectively). Treating NOFs with receptor antagonists to both receptors resulted in the greatest inhibition of proliferation of 30% in comparison to ET-1 treated NOFs.

5.3 ET-1 and Ang II promote migration of NOFs

When fibroblasts acquire a more activated phenotype, a common characteristic associated with the change is the ability of the cell to migrate more. A wound healing model was used to determine the effect of ET-1 on NOF migration. NOFs were seeded and allowed to grow until they reached confluency. The cells were serum starved overnight and the following day a 'scratch' was made through the confluent monolayer using a pipette tip (Figure 5.2A). The cells were washed and treated with ET-1 receptor antagonists or vehicle controls in serum free DMEM. Mitomycin C was added to the cells at the same time as the treatments in order to inhibit proliferation of the NOFs. The migration of the cells can thus be distinguished from their ability to proliferate into and reduce the 'scratch' diameter. The treatment of NOFs with ET-1 significantly increased the migration of the cells, almost to a similar extent as that observed in response to serum. ET-1 treatment of the NOFs resulted in a 90% increase in cell migration in comparison to untreated cells (Figure 5.2B). DMEM containing 10% (v/v) FBS was used within the experiment to act as a positive control. When the cells were treated with DMEM containing 10% (v/v) FBS, NOFs increased their migration by 100% in comparison to untreated cells. Pre-treatment of NOFs with BQ-123, and/or BQ-788, blocked the migration of the cells stimulated by ET-1 treatment. When the NOFs were pre-treated with the receptor antagonists to either ET_AR or ET_BR, a reduction in migration of 90% was observed, in comparison to cells treated with ET-1. This reduction was further increased to 100% when cells were pre-treated with both antagonists in combination. This result was also plotted in comparison to ET-1 treated cells.

The wound healing model was used again in order to determine the effect of Ang II on NOF migration (Figure 5.3A). In this experiment transforming growth factor-β (TGF-β) was used as a positive control (Leask and Abraham, 2004). The treatment of NOFs with TGF-β resulted in a 75% increase in NOFs migration in comparison to untreated cells (Figure 5.3B). Treatment with Ang II also resulted in an increase in the migratory nature of the NOFs. Ang II stimulated NOF migration by 50% compared to untreated cells. These findings suggest that the treatment

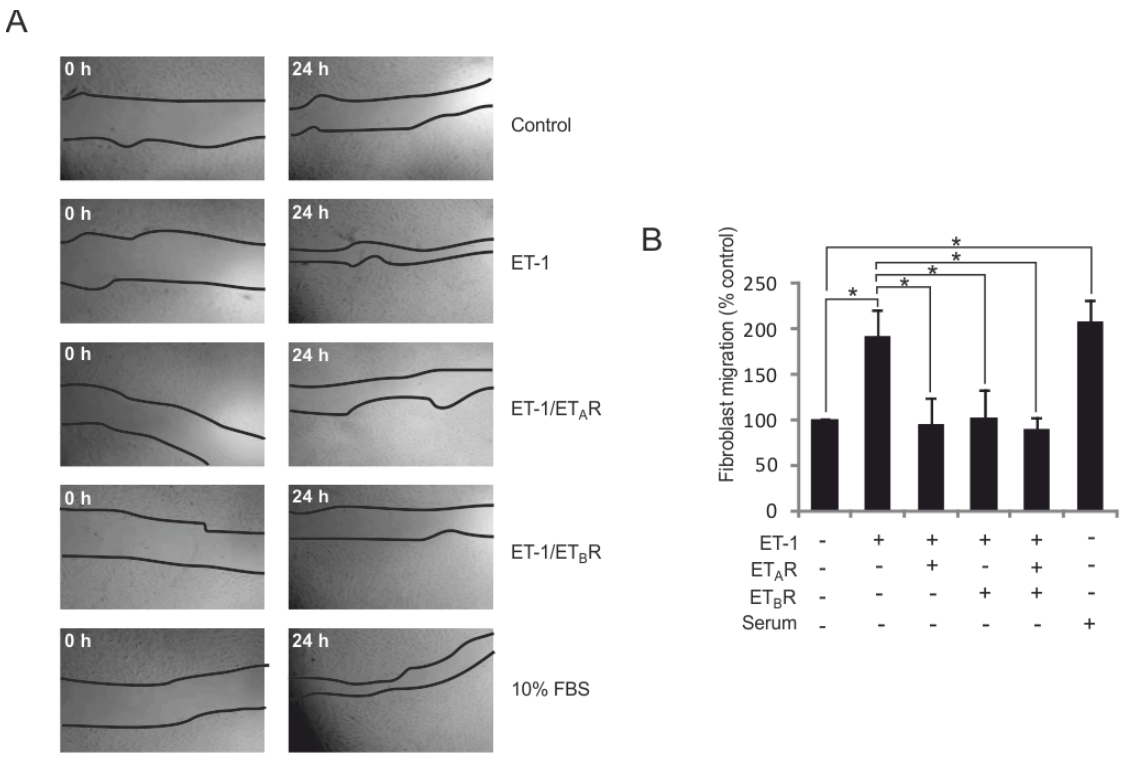


Figure 5.2 ET-1 stimulates NOF migration: Fibroblasts found within the stroma of the tumour microenvironment have been shown to have a more migratory phenotype. The migration of NOFs treated with ET-1 (10 nM) was assayed using a wound healing assay. Briefly, NOFs were cultured as a confluent monolayer in 12-well titre plates and allowed to adhere. A scratch was made through the serum starved cells using a pipette tip. The cells were pre-treated with a specific receptor antagonist to ETAR, BQ-123 (1 µM) and/or a specific receptor antagonist to ETBR, BQ-788 (1 µM) for 30 min before the addition of ET-1 (10 nM) or DMEM containing 10% (v/v) serum. Mitomycin C (1 µg/µl) was added to inhibit proliferation. Photographs were taken at two positions along the scratch at 0 h and 24 h time points **(A)** and the distance between the two edges of the scratch were measured **(B)** and the distance migrated by the cells was calculated. Each data point represents an average of at least 3 independent experiments, carried out in duplicate. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*) relative to untreated control, or as indicated by bars.

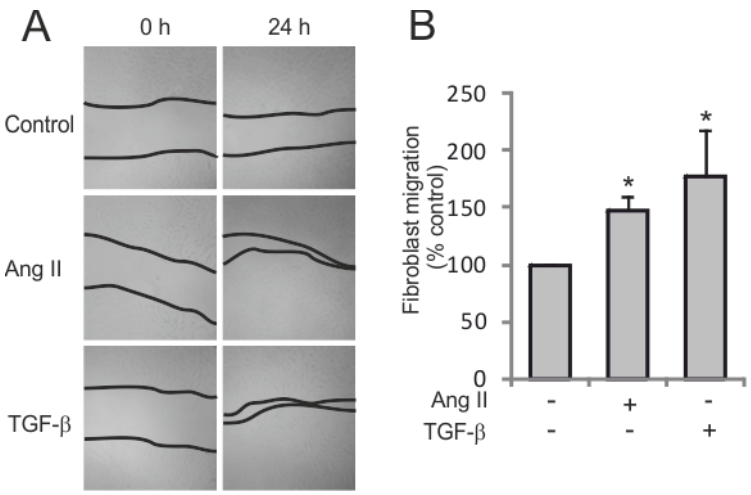


Figure 5.3 Ang II stimulates NOF migration: TGF-β is a well characterized stimulant of myofibroblast transdifferentiation. Migration of NOFs treated with Ang II (100 nM) was assayed using a wound healing assay. Briefly, NOFs were cultured as a confluent monolayer in 12-well titre plates and allowed to adhere. A scratch was made through the serum starved cells using a pipette tip. The cells were treated with Ang II (100 nM) or TGF-β (20 ng/µl). Mitomycin C (1 µg/µl) was added to inhibit proliferation. Photographs were taken at two positions along the scratch at 0 h and 24 h time points **(A)** and the distance between the two edges of the scratch were measured **(B)** and the distance migrated by the cells was calculated. Each data point represents an average of at least 3 independent experiments, carried out in duplicate. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*) relative to untreated control.

of NOFs with either the ET-1 or Ang II results in a phenotypic change in the cells, stimulating them to migrate more.

5.4 ET-1 and Ang II stimulate contraction of NOFs

When fibroblasts become 'activated' they acquire a more contractile phenotype. ET-1 is known to promote human skin fibroblast contraction (Guidry and Hook, 1991). The contraction of fibroblasts is an important action in the processes of wound healing and dermis reconstitution (Grinnel, 1994). ET-1 is also capable of modifying the ECM (Levin, 1995), increasing collagen I and III synthesis, and it can decrease the mRNA and protein levels of matrix mettaloproteinase-1 (MMP-1) in dermal fibroblasts (Xu *et al*, 1998; Shi-Wen *et al*, 2001). A fibroblast:collagen contraction model was used to determine the ability of ET-1 to stimulate the contraction of NOFs. NOFs were mixed with collagen and seeded into 24-well plates. After incubation overnight in serum free media the fibroblast:collagen gels were detached from the edges of each well and treated with ET-1. The ability of each gel to contract and decrease in size after 30 min was measured (Figure 5.4A). The decrease in gel size reflects the increased contractile capability acquired by the NOF cells. Treatment of fibroblast:collagen gels with ET-1 resulted in a decrease in gel size of 25% in comparison to fibroblast:collagen gels which were treated with serum free media only (Figure 5.4B). Thrombin is known to stimulate fibroblast contraction and was used as a positive control in this experiment (Fang *et al*, 2004). Treatment of fibroblast:collagen gels with thrombin resulted in a 30% reduction in their size in comparison to vehicle treated gels. In order to investigate through which receptor ET-1 was exerting its effects the gels were pre-treated with BQ-123 and BQ-788, both alone and in combination, for 30 min before treatment with ET-1. Treatment with BQ-123 resulted in contraction of the gels by only 10% and treatment with BQ-788 resulted in contraction similar to that observed when gels were treated with serum free media only. This reduction in contraction was further increased when both receptor antagonists were utilised together. The stimulation of a contractile phenotype in NOFs by ET-1 appeared to be predominantly mediated by ET_BR, with antagonism of ET_AR having a smaller effect.

The fibroblast:collagen models were also used to determine the effect of Ang II on the NOF contractile phenotype. The presence of the peptide resulted in a decrease in gel size of 40% in comparison to gels treated with serum free media only (Figure 5.5A). Thrombin was again used as a positive control within this experiment and treatment of the fibroblast:collagen gels with the enzyme reduced gel size to 40% compared to untreated cells, a similar observation to that observed with Ang II treatment.

Both peptides appear to be able to promote a more contractile phenotype in NOFs.

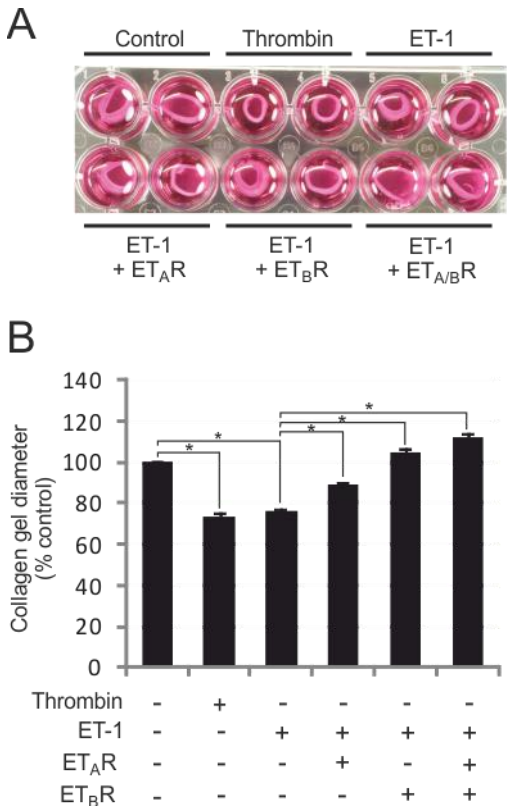


Figure 5.4 ET-1 invokes NOFs to have a more contractile phenotype: The acquisition of NOFs to a more activated state usually coincides with a more contractile phenotype. The contractile nature of NOFs when treated with ET-1 was assayed using a fibroblast:collagen contraction model. Briefly, NOFs were added to collagen and seeded into 24 well titre plates and allowed to incubate overnight. The fibroblast:collagen gels were detached from the edges of each well and pre-treated with specific receptor antagonists to ET_AR (1 µM) and/or ET_BR (1 µM) for 30 min before the addition of ET-1 (10 nM) or thrombin (0.5 units/ml). The distance each gel contracted was photographed **(A)** and the decrease in gel size after 30 min was measured **(B)**. Each data point represents an average of at least 3 independent experiments, carried out in duplicate. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*) relative to untreated control, or as indicated by bars.

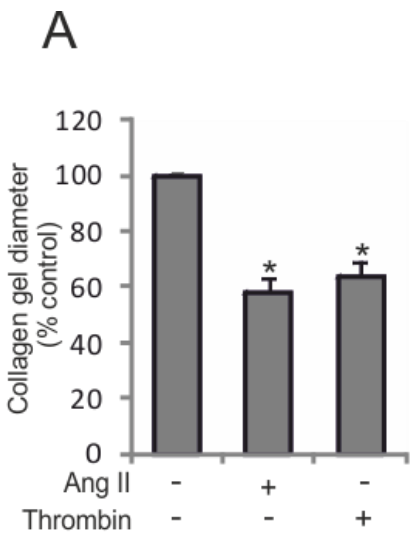


Figure 5.5 Ang II invokes NOFs to have a more contractile phenotype: The contractile nature of NOFs when treated with Ang II was assayed using a fibroblast:collagen contraction model. Briefly, NOFs were added to collagen and seeded into 24 well titre plates and allowed to incubate overnight. The fibroblast:collagen gels were detached from the edges of each well and treated with Ang II (100 nM) or thrombin (0.5 units/ml). The distance each gel contracted was photographed and the decrease in gel size after 30 min was measured **(A)**. Each data point represents an average of at least 3 independent experiments, carried out in duplicate. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*) relative to untreated control.

5.5 The effect of ET-1 and Ang II on myofibroblast transdifferentiation of NOFs

TGF-β is a well-characterized stimulant of myofibroblast transdifferentiation. Myofibroblast transdifferentiation can be characterised by the increase in expression of a number of proteins including α-smooth muscle actin (α-SMA) and MMP-2; a member of the MMP family which is responsible for the degradation of the ECM in normal physiological conditions including tissue remodelling and embryonic development and in disease including the process of metastasis and arthritis. Both α-SMA and MMP-2 are described as 'markers' of myofibroblasts (Sobral *et al*, 2011). TGF-β mediated transdifferentiation of NOFs into myofibroblasts not only increases the levels of α-SMA and MMP-2 within the cells but also results in their increased ability to promote cancer cell migration. The ability of treatment with ET-1 to trigger NOFs to acquire an activated, myofibroblast-like phenotype was explored. Within this experiment TGF-β was used as a positive control. The treatment of NOFs with the TGF-β resulted in an increase in the expression levels of α-SMA transcript (2.5-fold) (Figure 5.6A) and protein (2.5-fold) (Figure 5.6B) and MMP-2 transcript (1.7-fold) (Figure 5.6C) levels in comparison to vehicle treated cells. Treatment of NOFs with ET-1 however did not significantly increase the transcript levels of α-SMA or MMP-2. ET-1 treatment resulted in only a small increase of 1.1-fold in both α-SMA and MMP-2 transcript levels and a 1.3-fold increase in α-SMA protein levels. All fold changes were plotted relative to untreated cells.

The ability of Ang II to stimulate NOFs to acquire an activated, myofibroblast-like phenotype was next investigated. NOFs were again treated with TGF-β within the same experiment in order for the cytokine to act as a positive control. Unlike ET-1, Ang II treatment resulted in a significant increase in the transcript levels of both α-SMA (Figure 5.7A) and MMP-2 (Figure 5.7B) (1.6-fold and 1.4-fold in comparison to untreated cells, respectively). This result was similar to that observed when NOFs were treated with TGF-β. TGF-β stimulated an increase in the expression of α-SMA 2.5-fold and the expression of MMP-2 1.7-fold compared to cells left untreated. The increase in α-SMA transcript expression could be inhibited to only 0.6-fold by the pre-treatment of NOFs with an AT_1R antagonist, telmisartan (Figure 5.7C). This finding suggests that the receptor is critical in the ability of Ang II to promote myofibroblast transdifferentiation. SDS-Page and western blot analysis also showed that a small increase (1.2-fold) in α-SMA protein levels after treatment with Ang II was also observed (Figure 5.7D). Treatment with TGF-β resulted in an increase in α-SMA protein levels of 1.6-fold. Both results were plotted in comparison to cells left untreated.

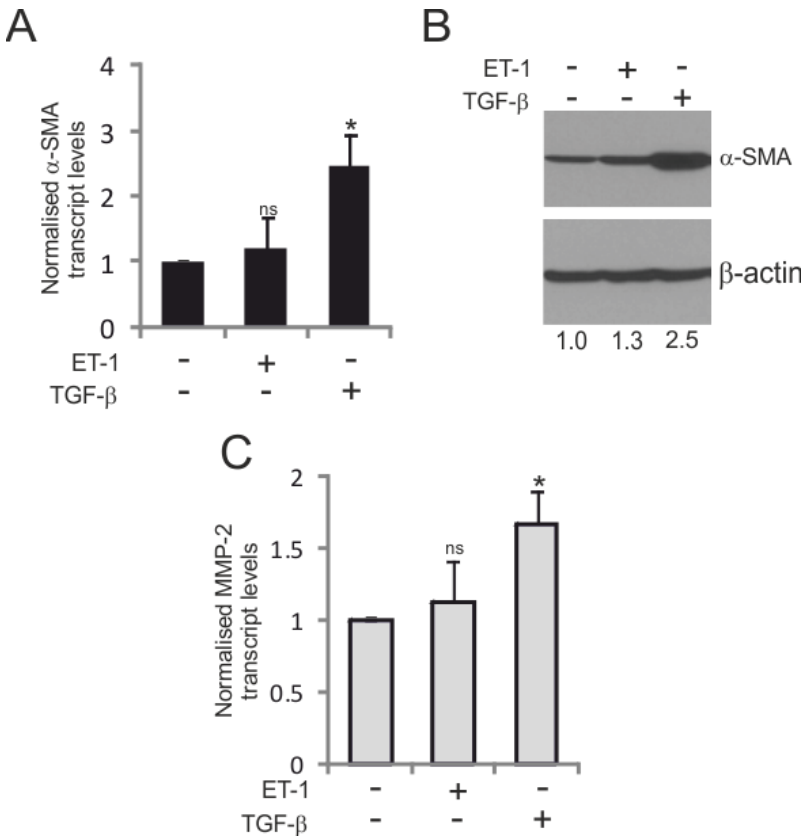


Figure 5.6 ET-1 does not stimulate markers of myofibroblast transdifferentiation: Myofibroblast transdifferentiation can be characterised by an increase in expression of α-SMA and MMP-2, a secretory component of the ECM, both of which are described as 'markers' of myofibroblasts. TGF-β is a well characterized stimulant of myofibroblast transdifferentiation and is known to increase expression of α-SMA and MMP-2. The ability of ET-1 to stimulate an increase in expression of α-SMA and MMP-2 in NOFs was assessed. Briefly, serum starved NOFs were treated with ET-1 (10 nM) or TGF-β (5 ng/µl) for 48 h before cells were harvested and RNA extracted or lysates prepared. qPCR analysis was used to measure the transcript levels of α-SMA **(A)**, MMP-2 **(C)** or U6 as a reference gene. Each data point represents an average of at least 3 independent experiments, carried out in duplicate. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*) relative to untreated control, ns = not significant. Cell lysates were separated by SDS-PAGE and immunoblotted for α-SMA and β-actin (as a loading control). A representative blot is shown **(B)** and the intensity of the band corresponding to α-SMA, determined by densitometry and normalized to β-actin levels in the same sample, is indicated under each lane.

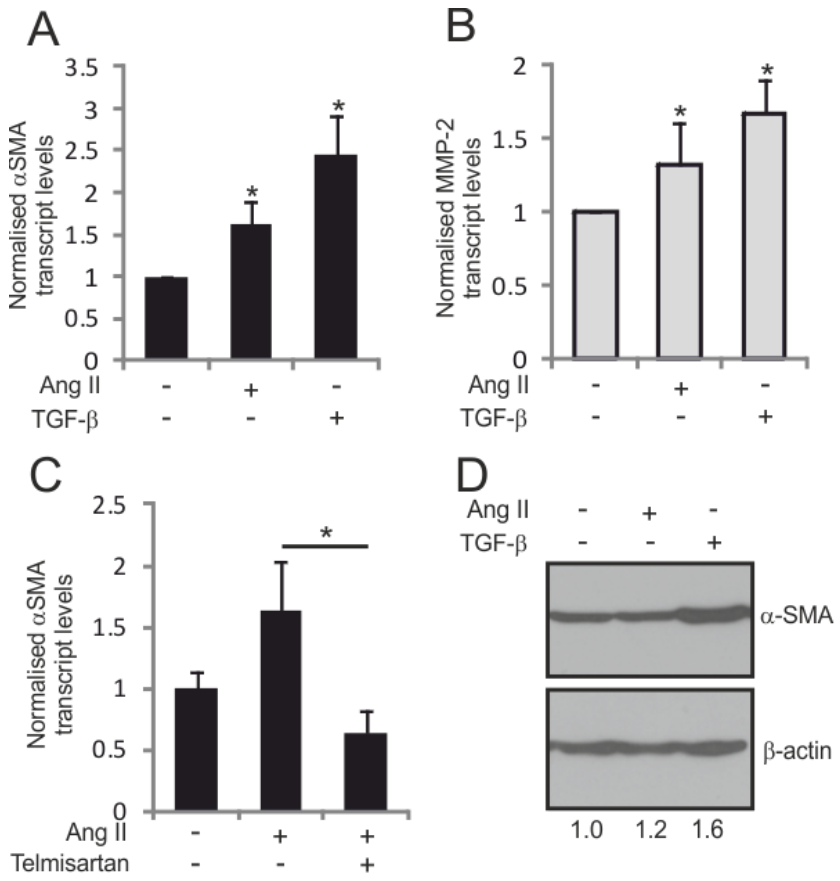


Figure 5.7 Ang II stimulates markers of myofibroblast transdifferentiation: The ability of Ang II to stimulate an increase in expression of α-SMA and MMP-2 in NOFs was assessed. Briefly, serum starved NOFs were treated with Ang II (100 nM) or TGF-β (5 ng/µl) for 48 h before cells were harvested and RNA extracted or lysates prepared. qPCR analysis was used to measure the transcript levels of α-SMA **(A)**, MMP-2 **(B)** or U6 as a reference gene. qPCR for α-SMA was also carried out on RNA extracted from NOFs pre-treated with telmisartan (100 nM), a specific AT_1R antagonist, for 30 min before the addition of 100 nM Ang II for a further 48 h **(C)**. Each data point represents an average of at least 3 independent experiments, carried out in duplicate. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*) relative to untreated control. Cell lysates were separated by SDS-PAGE and immunoblotted for α-SMA and β-actin (as a loading control). A representative blot is shown **(D)** and the intensity of the band corresponding to α-SMA, determined by densitometry and normalized to β-actin levels in the same sample, is indicated under each lane.

5.6 The combined treatment of NOFs with ET-1, Ang II and TGF-β can further potentiate paracrine and autocrine migration and myofibroblast transdifferentiation

Research conducted has identified that ET-1, Ang II and TGF-β can interact with each other to alter the behaviour of cells and the regulatory systems responsible for the control of the individual peptides (Lagares *et al*, 2010; Shi-Wen *et al*, 2007; Shephard *et al*, 2004; Alvarez *et al*, 2011). The synergistic effect of both peptides and the cytokine on the paracrine stimulation of HNSCC migration was therefore investigated. NOFs were treated with ET-1, Ang II and/or TGF-β alone or in combination for 4 h before the media was aspirated, filtered and added to the Transwell migration assay. The effect on the migration of serum starved SCC4 cells was observed. The treatment of NOFs with ET-1 resulted in an increase in SCC4 migration 1.8-fold in comparison to untreated NOFs (Figure 5.8A). Ang II treatment resulted in a similar increase in SCC4 migration, 1.6-fold greater than untreated NOFs. Treatment of NOFs with TGF-β stimulated SCC4 cells to migrate 2.0-fold compared to NOFs which were left untreated. The combined treatment of NOFs with ET-1 and Ang II resulted in an increase in SCC4 migration 2.1-fold greater than the effect on SCC4 migration stimulated by untreated NOFs. This however was not a significant increase in comparison to migration stimulated by ET-1 or Ang II treatment alone. There was a similar observation when NOFs were treated with ET-1 and TGF-β in combination. The combined treatment resulted in an increase in SCC4 migration of 2.3-fold but this was not significantly greater than each treatment alone. The combined treatment of NOFs with Ang II and TGF-β caused an increase in SCC4 migration 3.0-fold greater than untreated NOFs and this was a significant increase in SCC4 stimulated migration in comparison to Ang II treatment alone. It was however not significant in comparison to TGF-β alone. Finally NOFs were treated with ET-1, Ang II and TGF-β in combination. This treatment method resulted in an increase in SCC4 migration 3.7-fold greater than untreated NOFs. This further stimulation of SCC4 migration by combined treatment of all three peptides was significantly greater than the migration stimulated by NOFs treated with the three peptides individually.

A similar experiment was conducted to investigate the direct effect on NOF phenotype caused by combining the peptide treatments. A wound healing model was used to determine the effect of ET-1, Ang II and/or TGF-β alone or in combination on NOFs. Mitomycin C was again added at the same time as the treatment to inhibit NOF proliferation. Treatment of NOFs with ET-1 resulted in an 80% increase in their migrational ability compared to NOFs treated with serum free media only (Figure 5.9A). When NOFs were treated with Ang II, their migration increased 70%. TGF-β treatment stimulated their migration 60% more compared to cells that were left untreated. The treatment of NOFs with ET-1 and Ang II in combination resulted in a

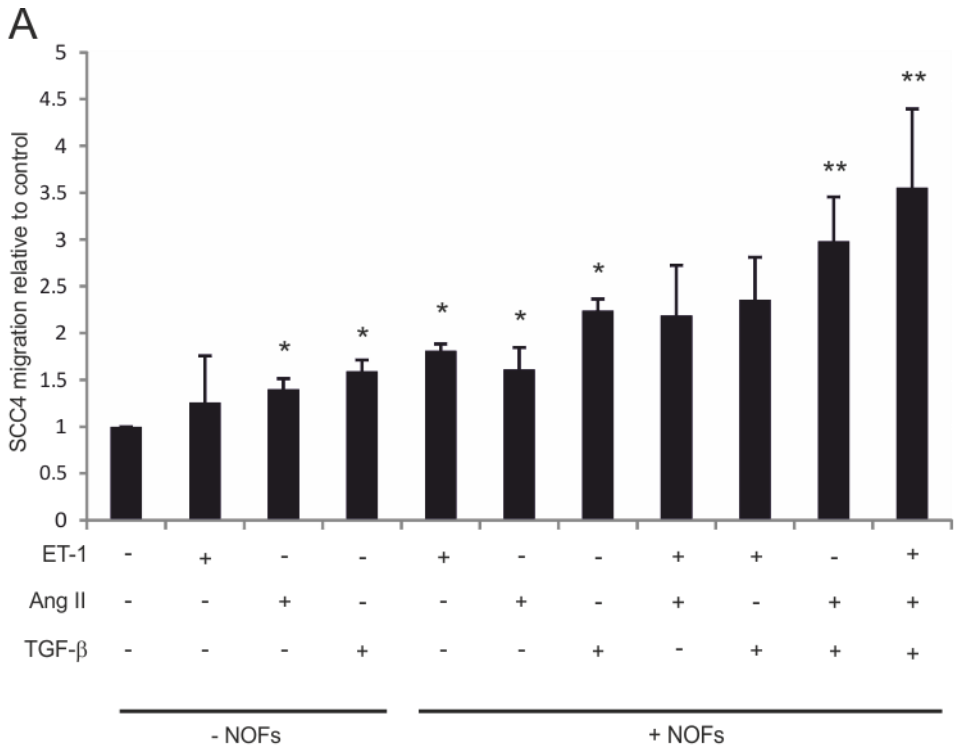


Figure 5.8 The combined treatment of NOFs with ET-1, Ang II and/or TGF-β can further potentiate paracrine stimulated HNSCC migration: NOFs were treated with ET-1 (10 nM), Ang II (100 nM) and/or TGF-β (5 ng/µL) alone and in combination for 4 h. Conditioned media was aspirated, filtered and added to the bottom Transwell migration well. Serum free media treated with ET-1 (10 nM), Ang II (100 nM) and/or TGF-β (5 ng/µL) was used as a control. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration inserts in DMEM supplemented with 0.1% (w/v) BSA. After 16 h, cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p<0.05$ relative to ET-1, Ang II or TGF-β only treated cells (**).

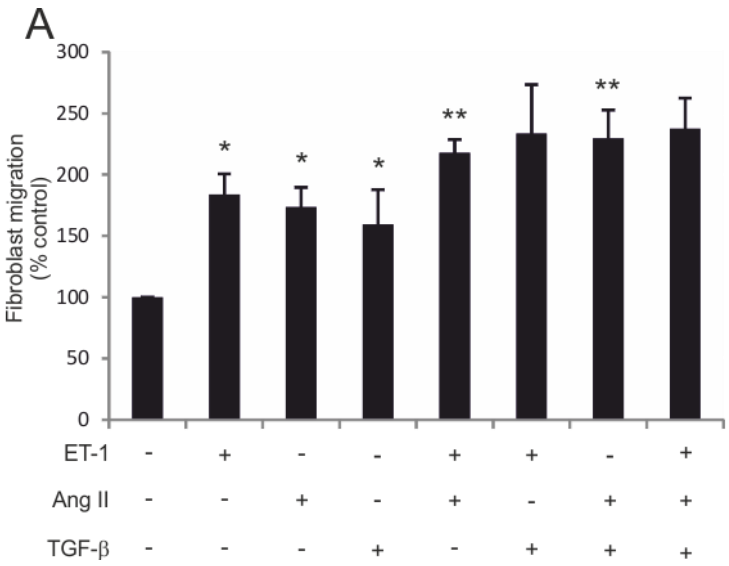


Figure 5.9 The combined treatment of NOFs with ET-1, Ang II and/or TGF-β can further potentiate autocrine stimulated migration: Briefly, NOFs were cultured as a confluent monolayer in 12-well titre plates and allowed to adhere. A scratch was made through the serum starved cells using a pipette tip. The cells were treated with ET-1 (10 nM), Ang II (100 nM) and/or TGF-β (5 ng/µl) alone or in combination. Mitomycin C (1 µg/µl) was added to inhibit proliferation. Photographs were taken at two positions along the scratch at 0 h and 24 h time points and the distance between the two edges of the scratch were measured (B) and the distance migrated by the cells was calculated. Each data point represents an average of at least 3 independent experiments, carried out in duplicate. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*) relative to untreated control, or as indicated by bars. $p<0.05$ relative to ET-1, Ang II or TGF-β only treated cells (**).

significantly greater increase in migration of 120% compared to NOFs treated with ET-1 or Ang II alone. The treatment of NOFs with ET-1 and TGF-β in combination and Ang II and TGF-β in combination produced similar results. Both combinations of treatments stimulated migration of NOFs 130% more compared to cells that were left untreated. The combined treatment of ET-1 and TGF-β only stimulated NOF migration significantly more than TGF-β treatment alone and not ET-1 treatment alone. The combined treatment with Ang II and TGF-β however did stimulate migration of the cells significantly more than the individual treatment with each peptide alone. Finally the treatment of NOFs with ET-1, Ang II and TGF-β stimulated NOF migration 140% more than the migration of untreated NOFs. This result was significantly greater than any increase in migration observed when cells with treated with each peptide individually.

The synergistic effect of ET-1, Ang II and/or TGF-β on myofibroblast transdifferentiation was investigated next. The effect on α-SMA transcript levels was used as a marker to determine the peptides and cytokines ability to promote transdifferentiation of the NOFs. NOFs were treated with ET-1, Ang II and/or TGF-β alone or in combination for 48 h. Treatment of NOFs with ET-1 did not result in an increase in α-SMA transcript levels in comparison to NOFs treated with serum free media only (Figure 5.10A). When NOFs were treated with Ang II there was a small increase in α-SMA transcript levels of around 1.2-fold compared to untreated cells. TGF-β treatment of NOFs resulted in a 2.5-fold increase in α-SMA transcript levels compared to untreated cells. The treatment of NOFs with ET-1 and Ang II in combination resulted in a greater increase in α-SMA transcript levels of 1.5-fold compared to NOFs treated with ET-1 or Ang II alone. The treatment of NOFs with ET-1 and TGF-β in combination resulted in a significantly greater stimulation of α-SMA transcript levels. ET-1 and TGF-β combined treatment increased transcript levels 4.2-fold compared to untreated NOFs. Ang II and TGF-β combined treated of NOFs produced similar results. This combined treatment resulted in an increase in α-SMA transcript levels of 3.1-fold compared to untreated cells. The treatment of NOFs with ET-1, Ang II and TGF-β stimulated α-SMA transcript levels 3.2-fold more than untreated NOFs. SDS-PAGE and western blot analysis also showed an increase in α-SMA protein levels when NOFs were treated with ET-1, Ang II and/or TGF-β in combination in comparison to the individual treatment of the cells with ET-1 or Ang II (Figure 5.10B).

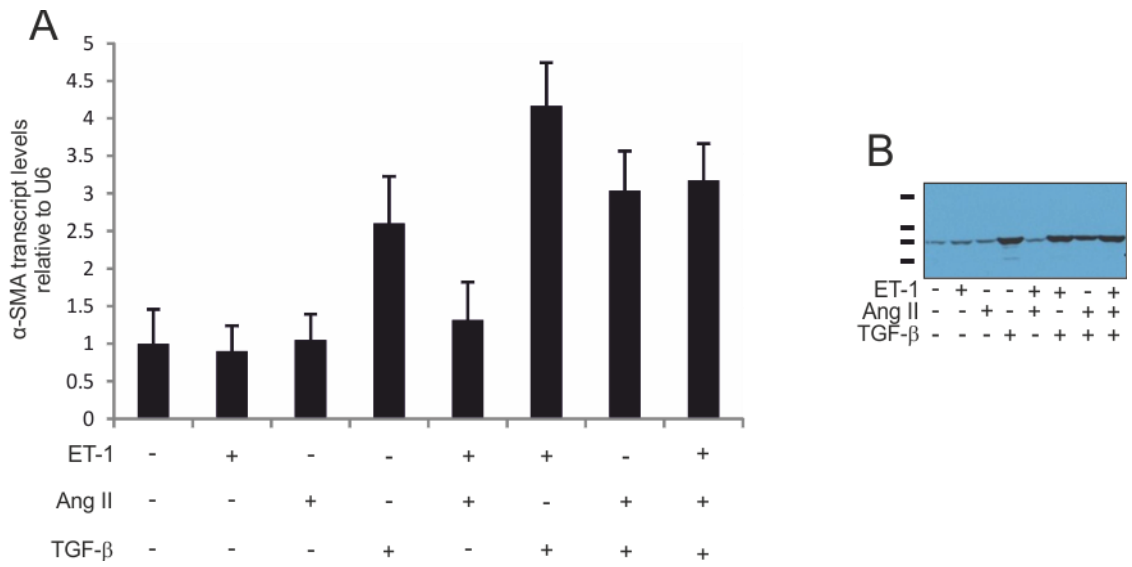


Figure 5.10 The effect of combining treatment of NOFs with ET-1, Ang II and/or TGF-β on α-SMA transcript and protein levels: NOFs were treated with ET-1 (10 nM), Ang II (100 nM) and/or TGF-β (5 ng/µl) alone or in combination for 48 h before the cells were harvested and RNA extracted or lysates prepared. qPCR analysis was used to measure the transcript levels of α-SMA **(A)** or U6 as a reference gene. Cell lysates were separated by SDS-PAGE and immunoblotted for α-SMA. A representative blot is shown **(B)**. Each data point represents an average of at least 3 independent experiments, carried out in triplicate. ± SEM are indicated.

5.7 Summary

Work conducted in this thesis has identified that both ET-1 and Ang II can promote tumour-stromal interactions between NOFs and HNSCCs resulting in an increase in the migration and invasion of the epithelial tumour cells. The aim of the work in this chapter was to elucidate the mechanism by which the mitogenic peptides are stimulating this communication network between the two cell types. Was the treatment of NOFs with ET-1 and Ang II resulting in a change in their phenotype? Fibroblasts found within the tumour microenvironment are known to undergo a phenotypic change in response to both factors found within the reactive stroma and those released from the nearby epithelial cancer cells. Work in this chapter was conducted in order to determine the phenotypic changes triggered by treatment of NOFs with both ET-1 and Ang II and to determine if they could stimulate the transdifferentiation of NOFs into myofibroblasts; a common characteristic associated with fibroblasts found within the reactive stroma of the tumour microenvironment. The treatment of NOFs with ET or Ang II resulted in an increase their ability to migrate, proliferate and contract; all of which are characteristics associated with a more 'activated' phenotype. ET-1, unlike Ang II however did not stimulate NOFs to transdifferentiate into myofibroblasts as indicated by its ability to not stimulate a significant increase in α-SMA transcript and protein levels and MMP-2 transcript levels. Both α-SMA and MMP-2 are deemed markers of myofibroblasts transdifferentiation. Ang II on the other hand did stimulate NOFs to transdifferentiate into myofibroblasts in part through the activation of the AT_1R.

The final section of this chapter included work that focused on the synergistic effects of both the ET-1 and Ang II mitogenic peptides and that of the TGF-β cytokine, a factor known to promote myofibroblast transdifferentiation. The combined treatment of ET-1, Ang II and/or TGF-β resulted in a further increase in the paracrine stimulation of HNSCC migration, NOF migration and α-SMA transcript levels suggesting that the peptides and cytokine can work together to further potentiate the effects triggered by individual treatment of NOFs with ET-1, Ang II and TGF-β alone.

Chapter 6: The identification of Ang 1-7 as a novel inhibitor of mitogenic peptide stimulated HNSCC migration and invasion

6.1 Introduction

Another arm of the renin angiotensin system (RAS) has recently been identified. Angiotensin 1-7 (Ang 1-7) is an endogenous, seven amino acid hormone that has both anti proliferative and vasodilator properties (Ferrario *et al*, 1997; Tallent *et al*, 1999; Santos *et al*, 2000). Ang 1-7 can be produced by the enzymatic breakdown of angiotensin I (Ang I) by a number of endopeptidases including neprilysin (NEP), prolyl oligopeptidase or thimet oligiopeptidase (Welches *et al*, 1991; Yamamoto *et al*, 1992; Chappel *et al*, 1995; Welches *et al*, 1993). This cleavage event takes place at the Pro-7-Phe-8 bond (Welches *et al*, 1993). It is considered that the production of Ang 1-7 is actually predominantly catalyzed by the angiotensin converting enzyme 2 (ACE2), which cleaves a single phenylalanine amino acid from the C-terminal of Angiotensin II (Ang II). It has been suggested that Ang 1-7 could have anti-proliferative effects, could act as a vasodilator and depressor (Ferrario *et al*, 1997), could inhibit angiogenesis (Machado *et al*, 2000; Machado *et al*, 2001) and have the ability to antagonize the effects triggered by Ang II (Herath *et al*, 2007). Treatment with ACE inhibitors, which result in an increase in Ang 1-7, have been linked to a reduction in the risk of cancer, particularly within the breast and lung (Lever *et al*, 1998). The ability of Ang 1-7 to oppose the profibrotic actions of Ang II in animal models of renal and cardiovascular disease has also been described. Ang 1-7 is thought to act in this instance through its receptor, Mas receptor (MasR) (Santos *et al*, 2003; Santos *et al*, 2008). Ang 1-7 treatment has also been shown to reduce transcript levels of endothelin-1 (ET-1) in cardiac fibroblasts (Iwata *et al*, 2005) and reduce the cerebral damage and behavioural deficits caused by ET-1 induced middle cerebral artery occlusion in a model of cerebral ischemia (Mecca *et al*, 2011). Both studies implicate that Ang 1-7 may inhibit the activity of other mitogenic peptides. Nothing is known of the role of Ang 1-7 in head and neck squamous cell carcinoma (HNSCC).

6.2 Ang 1-7 inhibits Ang II-induced autocrine cancer cell migration and invasion

The transcript levels of components of the RAS were examined in order to determine the ability of the oral mucosa to produce and/or respond to Ang 1-7. qPCR was used to determine the expression pattern of both ACE2 (Figure 6.1A) and the MasR (Figure 6.1B) in the same panel of primary human normal oral keratinocytes (NOKs) and normal oral fibroblasts (NOFs) and cell lines derived from oral dysplasias, primary HNSCCs and a local metastasis that was used previously and described in Table 2.2. ACE2 transcript levels were highest in NOKs and present at lower levels in cell lines derived from oral dysplasias, primary HNSCCs and a local metastasis. There was no expression of ACE2 detectable within the NOFs. The greatest levels of MasR transcripts were detectable within the different primary HNSCC cell lines. Transcripts

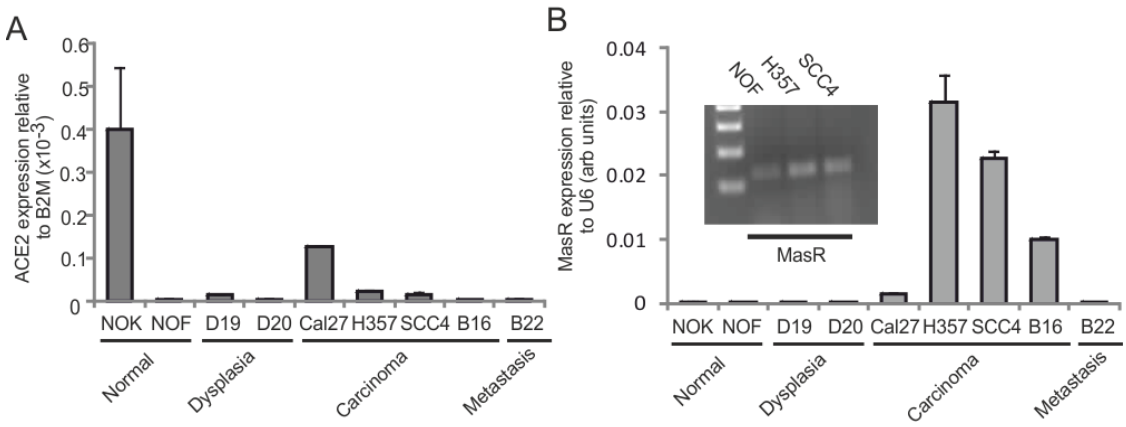


Figure 6.1 ACE2 and MasR expression: Ang 1-7 is produced from Ang II by the action of ACE2 (Herath et al, 2007). Ang 1-7 exerts its cellular effects via the MasR. Primary human NOKs and NOFs and cell lines derived from oral dysplasias, primary HNSCCs and a local metastasis were cultured in growth media and at 90% confluency were washed in PBS, trypsinised and pelleted. Total RNA was prepared from the cell pellets and 100 ng was subjected to RT-PCR. 0.25 ng cDNA was analysed using qPCR for ACE2 **(A)**, MasR **(B)**, B2M or U6 as a reference gene. RNA from NOFs, H357 and SCC4 were subjected to RT-PCR for MasR. Amplicons were separated by agarose gel electrophoresis and visualised by UV transillumination. Each data point represents an average of 3 technical repeats. ± SEM are indicated.

of the receptor were also detectable within NOKs, NOFs and cell lines derived from oral dysplasias and local metastasis but to a lower degree.

The ability of Ang 1-7 to influence HNSCC migration and invasion in the presence and absence of Ang II was next investigated. SCC4 cells were treated with Ang 1-7 alone and in combination with Ang II. The treatment of SCC4 cells with Ang 1-7 alone caused a small increase in cell migration (1.1-fold) in comparison to cells that were left untreated, however this increase was not significant (Figure 6.2A). The treatment of SCC4 cells with both Ang 1-7 and Ang II resulted in significant abrogation of Ang II stimulated SCC4 migration. Ang II alone stimulated cellular migration 1.5-fold in comparison to PBS vehicle treated cells. This was reduced to 0.8-fold when SCC4 were treated with Ang II and Ang 1-7 in combination. The same pattern of results was also observed for Ang II stimulated SCC4 invasion in the presence of Ang 1-7 (Figure 6.2B). The presence of the Ang 1-7 peptide resulted in a decrease in SCC4 invasion from 1.6-fold stimulated by Ang II treatment alone, to 0.9-fold compared to untreated cells. This reduction in cellular migration and invasion stimulated by Ang II treatment suggests that Ang 1-7 is able to have an inhibitory effect on the events triggered by Ang II.

In order to investigate if Ang 1-7 was causing its inhibitory effects via signalling through the MasR, SCC4 cells were treated with a specific MasR inhibitor, A-779. SCC4 cells were pre-treated with the inhibitor for 30 min before the addition of Ang 1-7 and/or Ang II. Treatment of SCC4 cells with Ang II alone resulted in a 1.5-fold increase in SCC4 migration (Figure 6.2C). The combined treatment of SCC4 cells with Ang II and Ang 1-7 decreased this level of migration to only 0.6-fold again highlighting the ability of Ang 1-7 to inhibit the effects triggered by Ang II. The presence of the MasR inhibitor, A-779 blocked this inhibitory effect and resulted in an increase in SCC4 migration of 1.4-fold in comparison to vehicle treated cells. All fold values were plotted in relation to PBS vehicle treated cells.

6.3 Ang 1-7 inhibits Ang II-induced paracrine cancer cell migration and invasion

The co-application of Ang 1-7 with Ang II significantly abrogates the ability of Ang II to promote HNSCC cell migration and invasion via an autocrine mechanism. The effect of exogenous Ang 1-7 on Ang II-stimulated paracrine signalling between NOFs and HNSCC cells was next investigated. As observed for direct application, treatment of SCC4 cells with conditioned media from NOFs incubated with Ang 1-7 and Ang II significantly inhibited the stimulation of cell migration and invasion observed in response to Ang II alone. The treatment of NOFs with Ang 1-7 alone resulted in a significant increase of 1.5-fold in comparison to vehicle treated

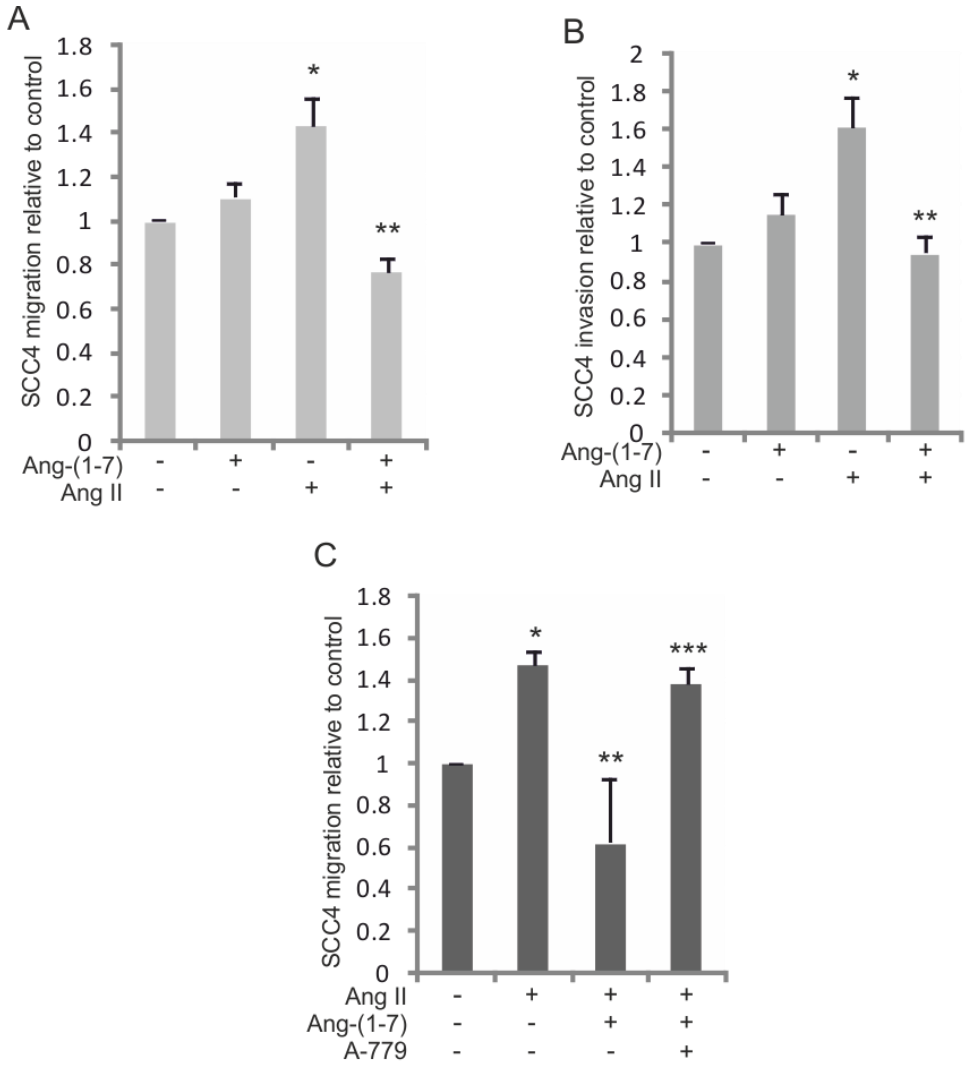


Figure 6.2 Ang 1-7 abrogates Ang II stimulated migration and invasion of HNSCC: The ability of Ang 1-7 to block Ang II stimulated migration and invasion of SCC4 cells was investigated. SCC4 cells were treated with Ang 1-7 (100 µM) and/or Ang II (100 µM) and assayed using a 2D Transwell migration assay **(A)** or a Matrigel invasion assay **(B)**. To investigate if Ang 1-7 is acting via the MasR in this instance, migration of SCC4 when pre-treated with a specific receptor antagonist to MasR, A-779 (100 µM) **(C)** for 30 min before the addition Ang II both alone and in combination with Ang 1-7 were assayed using a 2D Transwell migration assay. Briefly, $1x10^5$ serum starved SCC4 cells were treated as above and added to the top of the Transwell migration or Matrigel invasion inserts in DMEM supplemented with 0.1% (w/v) BSA with DMEM containing 10% (v/v) FBS placed at the bottom of the well. After 16 h (for migration) or 40 h (for invasion), cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent experiments. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p < 0.05$ relative to Ang II treated cells (**), $p < 0.05$ relative to Ang II and Ang 1-7 treated cells in combination (***).

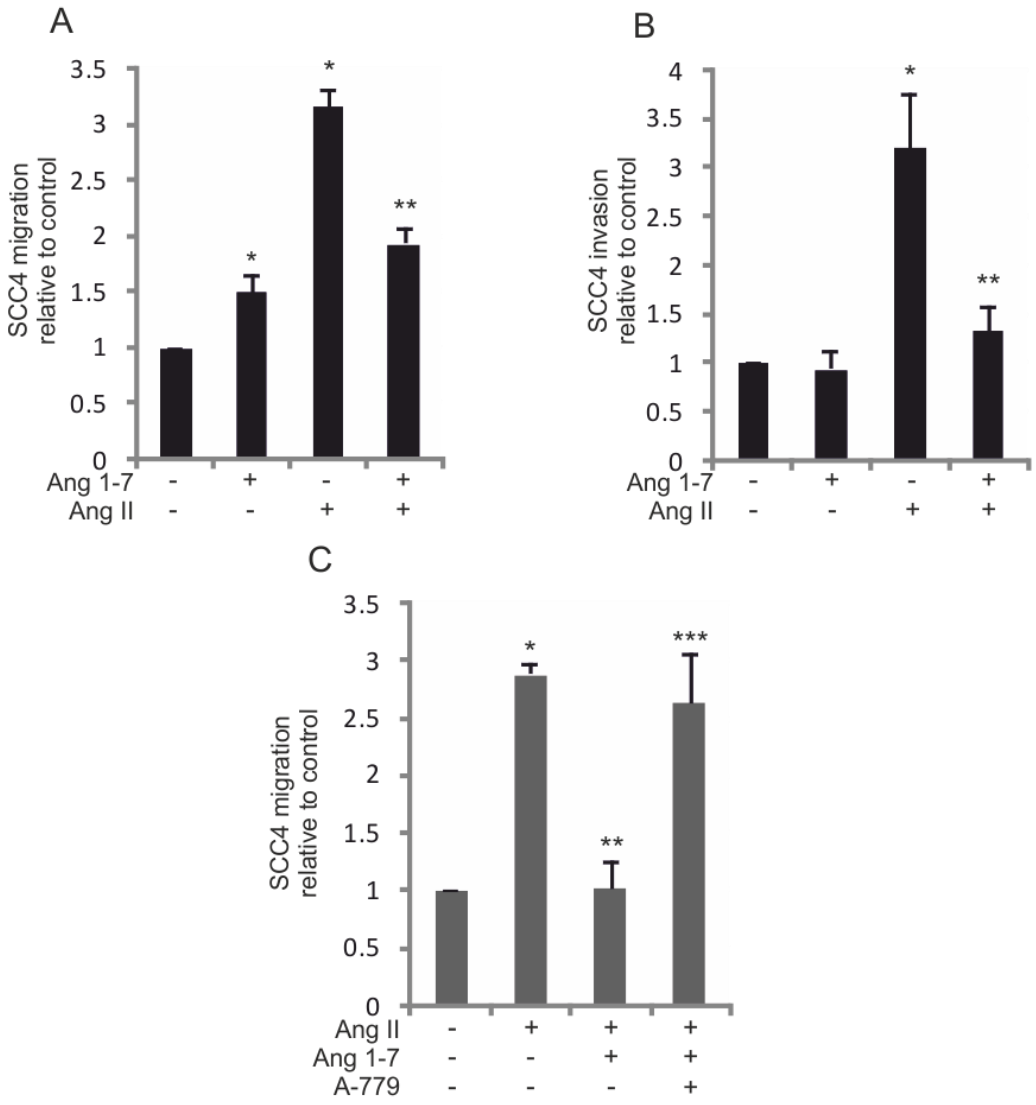


Figure 6.3 Ang 1-7 blocks the paracrine stimulation of HNSCC migration stimulated by Ang II: The ability of Ang 1-7 to inhibit paracrine stimulation by Ang II was investigated. NOFs were pre-treated with Ang 1-7 (100 nM) and/or a specific MasR antagonist, A-779 (1 µM) for 30 m before the addition of Ang II for a further 4 h. The conditioned media was aspirated, filtered and added to the bottom Transwell migration well **(A, C** -/+ A-779**)** or the bottom Matrigel invasion well **(B)**. 1x10^5 serum starved SCC4 cells were left untreated and added to the top of the Transwell migration or Matrigel invasion inserts in DMEM supplemented with 0.1% (w/v) BSA. After 16 h (for migration) or 40 h (for invasion), cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent observations. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p<0.05$ relative to Ang II treated cells (**), $p<0.05$ relative to Ang 1-7 and Ang II treated cells in combination (***).

NOFs, in SCC4 migration (Figure 6.3A). Ang 1-7 however, did not stimulate SCC4 invasion under the same experimental circumstances. As previously observed, Ang II stimulated migration of SCC4 cells 3.1-fold and invasion of SCC4 cells 3.0-fold. This was reduced in both circumstances to 1.5-fold for migration and invasion when NOFs were treated with both Ang 1-7 and Ang II (Figure 6.3A and 6.3B, respectively). Each fold change was plotted in relation to cells lefts untreated within each individual experiment. To determine if Ang 1-7 was inhibiting Ang II stimulated SCC4 migration and invasion through the MasR, the MasR-specific antagonist, A-779 was used. NOFs were pre-treated with the receptor antagonist for 30 min before the addition of Ang 1-7 and Ang II. Prior incubation with A-779 again reversed this inhibition, a similar result to that observed for autocrine Ang II-stimulated HNSCC migration and invasion. In this experiment treatment of NOFs with Ang II stimulated SCC4 migration 2.8-fold in comparison to vehicle treated cells (Figure 6.3C). This was reduced to 1.0-fold with combined treatment with Ang II and Ang 1-7. Pre-treatment with A-779 returned this value to around 2.5-fold.

6.4 Ang 1-7 can also block the paracrine stimulation of HNSCC migration by ET-1 and BK but not EGF

Having established that Ang 1-7 can abrogate Ang II-induced paracrine effects on SCC4 migration, the signalling mechanisms involved were next investigated. Results within this thesis have identified that Ang II can promote the release of stimulatory factors by activating members of the ADAMs family of cell surface proteinases. These factors include heparin bound-epidermal growth factor (HB-EGF), tumour growth factor-α (TGF-α) and amphiregulin; ligands of the EGF receptor (EGFR). In order to examine whether Ang 1-7 was mediating its effects by interfering with this stimulation of ligand release, the ability of Ang 1-7 to reduce the activation of HNSCC migration provoked by other peptides was investigated. NOFs were treated for 4 h with Ang 1-7 combined with Ang II, ET-1, bradykinin (BK) and/or EGF which stimulated EGFR directly. After 4 h the media was removed, filtered and added to the bottom well of a Transwell migration assay. Untreated SCC4 cells were added to the top chamber of the assay and their ability to migrate investigated. After 16 h the migrated SCC4 cells were fixed, stained and counted using the method described in Section 2.2.4. The presence of conditioned media collected from NOFs treated with Ang 1-7 and Ang II, Ang 1-7 and ET-1 and Ang 1-7 and BK all caused a significant decrease in SCC4 cell migration (0.8-fold, 3.7-fold and 1.8–fold, respectively in comparison to 3.4–fold, 5.6-fold and 2.8–fold with Ang II, ET-1 and BK treatment alone) (Figure 6.4). The combined treatment of NOFs with Ang 1-7 and EGF did not inhibit SCC4 paracrine migration in comparison to EGF treatment alone (3.4-fold vs. 3.5–fold, respectively). Each fold change was plotted in comparison to cells left untreated.

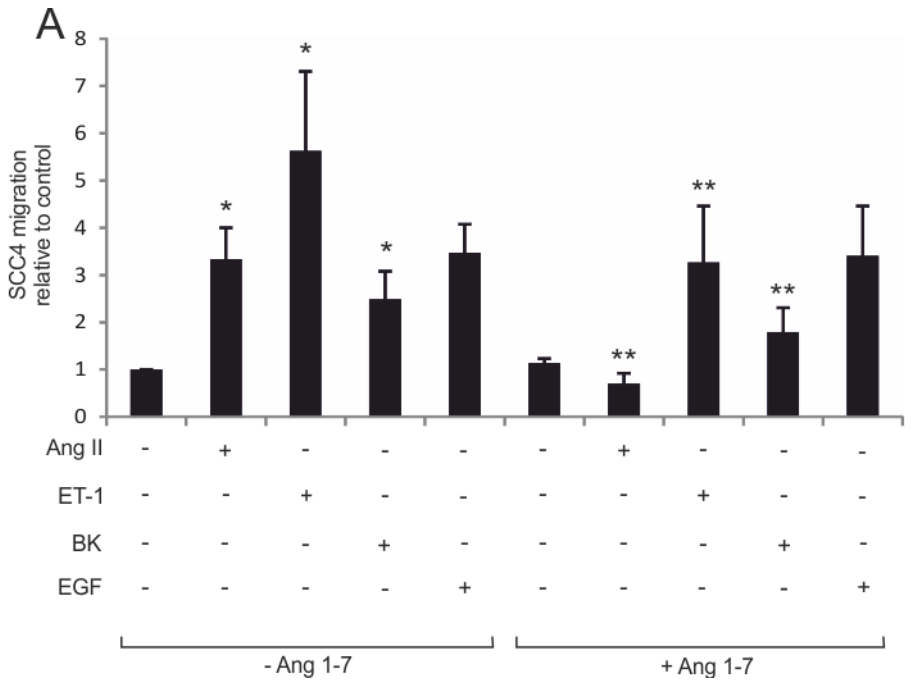


Figure 6.4 Ang 1-7 blocks the paracrine stimulation of HNSCC migration stimulated by mitogenic peptides: NOFs were pre-treated with Ang 1-7 (100 nM) for 30 m before the addition of Ang II (100 nM), ET (10 nM), BK (1 µM) and/or EGF (48.4 µM) alone or in combination for a further 4 h. The conditioned media was aspirated, filtered and added to the bottom Transwell migration well. $1x10^5$ serum starved SCC4 cells were left untreated and added to the top of the Transwell migration in DMEM supplemented with 0.1% (w/v) BSA. After 16 h cells which migrated to the underside of the Transwell permeable membrane were fixed in 100% (v/v) methanol. Migrated cells were stained using crystal violet (0.1% (w/v)) and counted using a light microscope. Data plotted represent average number of cells which migrated relative to untreated control and were calculated from an average of 3 fields of view. Each data point represents an average of at least 3 independent observations. ± SEM are indicated. A two-tailed student *t*-test was carried out and data deemed statistically significant when $p<0.05$ (*), $p<0.05$ relative to Ang II, ET-1, BK or EGF only treated cells (**).

6.5 Summary

This chapter has identified that a recently identified effector peptide of the RAS, Ang 1-7 can inhibit the effects on HNSCC migration and invasion stimulated by Ang II treatment. Ang 1-7 is a small peptide that is thought to be able to inhibit the effects triggered by Ang II. Ang 1-7 is produced from Ang II via the catalytic activity of ACE2 and the peptide has been identified as a ligand for the MasR. qPCR analysis identified the expression pattern of both components of the RAS, ACE2 and MasR within a panel of primary cells and cell lines. Both components are found within all cell types apart from ACE2 which is not detectable within NOFs. This result suggests that Ang 1-7 can be produced at a local level within the tumour microenvironment. Within this chapter experiments have indicated that the treatment of both HNSCCs and NOFs with Ang 1-7 results in the inhibition of migration and invasion triggered by stimulation with Ang II. Inhibitors to the MasR block the antagonising effect of Ang 1-7 on Ang II stimulated autocrine and paracrine activity suggesting that the peptide is exerting its effects through this receptor. Ang 1-7 can also inhibit the paracrine stimulation of HNSCC cell migration triggered by the treatment of NOFs with other mitogenic peptides including ET-1 and BK. This suggests that Ang 1-7 does not inhibit Ang II activity exclusively and this finding fuels the possibility that the peptide could be used as a novel therapeutic application in the treatment of head and neck cancer

Chapter 7: Discussion

7.1 Introduction

Head and neck cancer is the 6th most common malignancy worldwide and contributes to 3% of all cancers diagnosed in the United Kingdom (UK) each year (Cancer Research UK, 2012). Head and neck cancer has a five-year survival rate of around 50% and little improvement has been made in this figure over the past three decades (Cancer Research UK, 2012). The main treatment options are surgery and courses of radiotherapy (Scully and Porter, 2000) and an early diagnosis, prior to metastatic spread, is vital for a good prognosis (La Vecchia *et al*, 1997, Woolgar, 1999). The prognosis of head and neck cancer is also affected by the positioning of the lesion within the mouth (La Vecchia *et al*, 1997). Compared to some more studied cancers, little is known about the mechanisms underlying head and neck cancer progression and there is an urgent need for this to be addressed and novel therapeutic strategies to be developed.

Mitogenic peptides have been implicated in the progression of many types of cancer. Their expression and that of the enzymes controlling their levels are known to alter during tumourigenesis. Bradykinin (BK), endothelin-1 (ET-1) and angiotensin II (Ang II) are three examples of mitogenic peptides. Although the role of BK has been extensively studied in head and neck carcinogenesis (Thomas *et al*, 2006; Zhang *et al*, 2008), little is known of the role of ET-1 or Ang II.

7.2 The role of the ET-axis in promoting head and neck cancer progression via an autocrine mechanism

ET-1 is 21 amino acids in length and acts primarily as a potent endogenous vasoconstrictor (Yanagisawa *et al*, 1988). ET-1 is the product of the cleavage of pre-pro ET-1 to big ET-1 by a furin-like endopeptidase, and then the cleavage of big ET-1 to ET-1 by endothelin converting enzyme-1 (ECE-1), a membrane-bound metalloproteinase (McMahon *et al*, 1991; Rubanyi and Polokoff, 1994). ET-1 is the main peptide of the ET-axis. The ET-axis not only consists of ET-1 but also the two G protein-coupled receptors (GPCRs) through which the peptide can exert its effects; ET_AR and ET_BR. The ET-axis also includes the enzyme responsible for the activation of ET-1; ECE-1. ECE-1 is a type II membrane-bound zinc metalloprotease and is a member of the neutral endopeptidase family. It has four distinct isoforms: ECE-1a, ECE-1b, ECE-1c and ECE-1d all of which are derived from a single gene through the use of alternative promoter regions. Neprilysin (NEP) is another zinc metalloprotease and a member of the neutral endopeptidase family. NEP is important in regulating signal transduction processes throughout the cell and it can inactivate mitogenic neuropeptides including ET-1. NEP is known to be down-regulated in cancers, including metastatic human prostate cancer where it has been implicated in the transition from the androgen-dependent form of the disease to the more severe androgen-

independent form (Nelson *et al*, 2003). In head and neck squamous cell carcinoma (HNSCC), levels of the ET-1 mitogenic peptide are elevated in the saliva of patients suffering with the disease (Pickering *et al*, 2007). The presence of the peptide correlates with a poor prognosis and Awano *et al* (2006) have shown that HNSCCs produce more ET-1 and have identified that treatment of the cell lines with ET-1 results in an increase in their proliferation. The ET-axis has been implicated in HNSCC however the molecular mechanisms underlying its role within this environment remain unclear. The use of therapeutic agents to the ET-axis in the treatment of other cancers (Bhalla *et al*, 2009) raises the possibility that the components of the axis could provide new targets in the treatment of HNSCC.

Treatment of SCC4 cells, a HNSCC cell line isolated from the tongue, with ET-1 resulted in small but insignificant increase in cell migration. Awano *et al* (2006) identified that ET-1 can stimulate the proliferation of HNSCC cells via an autocrine mechanism, suggesting ET-1 may contribute to tumour growth. In this thesis the effect of ET-1 on HNSCC proliferation was not examined but the effect on migration, another hallmark associated with cancer progression, was. ET-1 did not significantly stimulate HNSCC migration therefore suggesting that the peptide uses different underlying autocrine mechanisms to promote HNSCC proliferation and migration.

Few studies have looked at the molecular mechanism underlying the role that ET-1 plays in the promotion of head and neck cancer progression. There is a need to investigate these mechanisms and to determine which cellular processes including, migration and invasion, ET-1 affects in order to contribute to the process of HNSCC progression. In the work presented in this thesis, qPCR analysis was used to investigate the expression of the components of the ET-axis within a panel of primary human normal oral keratinocytes (NOKs) and normal oral fibroblasts (NOFs) and cell lines derived from oral dysplasias, primary HNSCCs and a local metastasis. This study would determine if critical components of the ET-axis are present within the head and neck cancer microenvironment. Pre-pro ET-1 mRNA was expressed in all cell types but no significant trend in differential expression was observed. ECE-1 transcript levels were expressed to a greater extent in NOFs and in cell lines isolated from primary HNSCCs, oral dysplasias and a local metastasis in comparison to NOKs. Protein analysis on the same panel of cells also confirmed this increase. ECE-1 is responsible for the production, action or degradation of a number of catalytically active peptides including ET-1, BK, neurotensin, atrial natriuretic factor and substance P (Shipp *et al*, 1991; Shipp and Look, 1993). Its high expression within primary HNSCCs, oral dysplasias and local metastasis in comparison to NOKs could therefore result in the production of more catalytically active peptides, or potentiate their action, promoting cancer progression. NEP was highly expressed

within the NOFs and a reduction in NEP transcript levels was observed in primary HNSCCs cell lines both in comparison to NOKs. The reduction in NEP expression in primary HNSCC cell lines is reminiscent of that observed in prostate cancer (Nelson *et al*, 2003). Androgen-dependent metastatic prostate cancer cells express NEP and the progression to the androgen-independent form of the disease coincides with a reduction in the expression levels of the enzyme therefore allowing the peptides it normally inactivates, such as ET-1, to stimulate cell proliferation. A loss of NEP expression is associated with a number of malignancies. These include cancers of the lung, stomach, bladder, renal and prostate (Gohring *et al*, 1998; Koiso *et al*, 1994; Sato *et al*, 1996; Cohen *et al*, 1996; Pekonen *et al*, 1995; Papandreou *et al*, 1998). The loss of NEP activity results in the build up of mitogenic peptides at the cell plasma membrane which leads to the promotion of cellular proliferation (Papandreou *et al*, 1998). The results obtained here suggest a similar mechanism could be occurring in HNSCC. The loss of NEP expression, combined with the increased expression of ECE-1 observed in cell lines isolated from primary HNSCCs, could result in an increase in peptide production or activity at the plasma membrane of oral epithelial cells which would normally be controlled by the catalytic activity associated with NEP. Further work needs to be conducted in order to fully elucidate the role of NEP in head and neck cancer progression.

ET-1 can exert its effects via one, or both of its receptors, ET_AR and ET_BR, both of which are components of the ET-axis. The predilection of ET-1 for either receptor appears to vary depending upon context. ET-1 promotes ovarian and prostate cancer progression via ET_AR however in melanoma the interactions between the peptide and ET_BR seem to be more important (Nelson *et al*, 2003). qPCR analysis was used to determine the expression of both receptors within the same panel of human primary NOKs and NOFs and cell lines derived from primary carcinomas, dysplasias and a local metastasis used previously. ET_AR transcript levels were highly expressed in NOFs and were present in all other cell types. ET_BR transcripts were also readily detectable in NOFs but were reduced in HNSCC cell lines and the local metastasis, B22. This is in keeping with previous findings that the receptor is reduced in a number of HNSCC cell lines (Viet *et al*, 2011). The activation of the ET_BR has been associated with the clearance of ET-1, via its endocytosis and the triggering of cell death and apoptosis (Khodorova *et al*, 2003; Dupuis *et al*, 2000; Peters *et al*, 2004). The reduction in its expression, that is associated with a number of pathophysiological conditions including cancer, may therefore result in an increase in biologically active ET-1 and therefore the promotion of events triggered by its activity mediated by ET_AR (Khodorova *et al*, 2003; Dupuis *et al*, 2000; Peters *et al*, 2004). Studies have suggested that the methylation of the ET_BR gene is responsible for the

silencing of the receptor in cancer (Piovezan *et al*, 2000; Jeronimo *et al*, 2003; Knight *et al*, 2009; Lo *et al*, 2002).

The increase in ECE-1 expression in primary HNSCCs, oral dysplasias and a local metastasis in comparison to NOKs raises the question that the enzyme may play a fundamental role in the progression of head and neck cancer. Preliminary work in this thesis was conducted in order to begin to further understand the role that the enzyme plays in the progression of head and neck cancer and the autocrine mechanisms via which it exerts its effects. Heterologous over-expression of ECE-1 in SCC4 cells resulted in an increase in cellular migration. This result could suggest that the presence of the enzyme after transient transfection results in an increase in the production of biologically active ET-1 that in turn can go on to stimulate the migration of HNSCC cells via an autocrine mechanism. The treatment of SCC4 with exogenous ET-1 in a previous experiment however did not stimulate a significant increase in HNSCC migration. This therefore suggests that ECE-1 under these conditions is not exerting its effects via this mechanism.

ECE-1 is a highly glycosylated, type II membrane-associated neutral metalloendopeptidase (Shimada *et al*, 1994) and is involved in a number of complex signalling mechanisms in order to maintain its tight regulatory control of peptides (Turner and Tanzawa, 1997). ECE-1 has a specific characteristic in that its different isoforms, which continuously traffic between the plasma membrane and endosomes, are pH sensitive and therefore degrade different neuropeptides at different locations depending on the particular pH within that location (Padilla *et al*, 2007; Roosterman *et al*, 2008; Johnson *et al*, 1999). ECE-1 can hydrolyse big-ET-1 to produce biologically active ET-1 at a neutral pH within the extracellular fluid (Takahashi *et al*, 1993; Hoang and Turner, 1997). The enzyme can also hydrolyse other peptides including angiotensin I (Ang I), neurotensin, substance P and BK at an acidic pH which is similar to that found within the endosomes (Hoang and Turner, 1997; Johnson *et al*, 1999; Fahnoe *et al*, 2000). The ability of the enzyme to hydrolyse different peptides at different pHs explains why ECE-1 preferentially hydrolyses big-ET. The affinity that ECE-1 also has for ET-1 is greater than that that it shows towards other peptides therefore further supporting its favoured activity towards big-ET. These explanations therefore indicate that although ECE-1 can hydrolyse BK within the head and neck tumour microenvironment, it is a rare event and therefore the peptide can stimulate HNSCC via an autocrine mechanism which results in an increase in prostaglandin E2 (PGE_2) production (Thomas *et al*, 2006).

As mentioned previously ECE-1 can hydrolyse neurotensin. Levels of the peptide and the receptor that it exerts its effects through, the neurotensin receptor 1 (NTSR1), are elevated in

HNSCC samples and are associated with metastatic spread and HNSCC progression (Shimizu *et al*, 2008). The increased expression of neurotensin and NTSR1 in HNSCC is associated with an increase in interleukin 8 (IL-8) and matrix metalloproteinase-1 (MMP-1) transcripts within the samples (Shimizu *et al*, 2008). MMPs are a group of enzymes responsible for the remodelling and destruction of the extra cellular matrix (ECM), a process which is critical in tumour progression (Coussens *et al*, 2002). The destruction of the basement membrane by MMPs allows tumour cells to invade into the surrounding connective tissue, entry and exit into and out of blood vessels and therefore aid metastasis to distant locations (Coussens *et al*, 2002). MMPs are known to be up-regulated in many cancers (Egeblad and Werb, 2002) and have therefore been extensively investigated as possible therapeutic targets in the treatment of cancer (Coussens *et al*, 2002). An increase in MMP-1 transcript production could therefore provide evidence for the increase in migration of HNSCC cells observed in this study. The over expression of ECE-1 could increase NTSR1 recycling back to the plasma membrane, resulting in the amplification of the intracellular signalling activated by the binding of the neurotensin ligand to its receptor.

A specific example of how ECE-1 regulates the activity of peptides involves the interference of the enzyme with the extracellular signal-regulated kinases (ERK) signalling pathway. ERK signalling can promote intracellular processes including cell proliferation and cell growth. ECE-1 is responsible for the complex control of ERK signalling associated with the activation of the neurokinin-1 receptor (NK1R) by substance P. NK1R has recently been identified as being over expressed in HNSCC which could further implement the activity of ECE-1 in the progression of head and neck cancer (Brener *et al*, 2009). In physiological conditions substance P binds to and activates the NK1R. NEP is responsible for the degradation of substance P within the extracellular fluid resulting in the inhibition of NK1R activation which triggers its proinflammatory actions (Okamoto *et al*, 1994; Lu *et al*, 1997; Sturiale *et al*, 1999). This process involves GPCR signal transduction which is implicated in autocrine signalling. GPCR signal transduction is controlled by regulating the concentrations of extracellular agonists and the coupling of the receptor to heteromeric G proteins (Cottrell *et al*, 2009). Although these control mechanisms are in place, GPCRs can be internalised and endocytosed. Within the endosomes the receptor can continue to signal via G protein independent mechanisms (Cottrell *et al*, 2009). β-Arrestins can mediate the internalisation of GPCR and their trafficking to intracellular endosomes where they then facilitate intracellular signalling of the GPCR (Cottrell *et al*, 2009). The mechanism via which β-Arrestins mediate this process include the coupling of the GPCR to clathrin and the clathrin adaptor AP2, which allows the receptor to be endocytosed (Ferguson *et al*, 1996; Goodman *et al*, 1996). This process is enhanced by the

phosphorylation of the GPCR kinases which increase the affinity of the GPCR for the β-Arrestins (Pelayo *et al*, 2011). The β-Arrestins translocate from the cytosol to the plasma membrane in order to uncouple the GPCR from the heterotrimeric G proteins. This action ultimately desensitises G protein-dependent signalling (Pelayo *et al*, 2011). The β-Arrestins can act as scaffolds which facilitate the recruitment of mitogen-activated protein kinases (MAPKs), Raf-1 and Scr to the GPCR forming a MAPK signalsome. This process and formation of the signalsome determines the location and activation of ERKs (DeFea *et al*, 2000; DeFea *et al*, 2000; Lutterell *et al*, 1999; Tohgo *et al*, 2002). Cottrell *et al* (2009) identify that ECE-1 can degrade neuropepetides including substance P, found within endosomes and that therefore regulate the formation, stability and activation of the signalsome which results in alterations in ERK signalling and subsequent activation of transcription factors.

ECE-1 destabilises the signalsome and therefore inhibits ERK signalling by degrading substance P rapidly within the endosome due to the acidic environment found within the cellular compartment. The pH within the extracellular fluid is neutral and at this pH ECE-1 degrades substance P more slowly (Cottrell *et al*, 2009). The acidic pH conditions found within the endosome could promote an increased rate of substance P degradation by increasing ECE-1 activity (Fahnoe *et al*, 2000) or the rate at which substance P dissociates from the NK1R (Grady *et al*, 1995; Roosterman *et al*, 2007). The disruption of the signalsome complex results in not only the degradation of substance P, but also allows the β-Arrestins to return to the cytosol, the NK1R is released from the β-Arrestins and can be recycled and resensitised at the cell surface (Roosterman *et al*, 2007). The recycling of the NK1R is required for sustained signalling and may also be responsible for the control of β-Arrestin-dependent mitogenic signalling of endocytosed receptors (Roosterman *et al*, 2007). The ECE-1b and ECE-1d isoforms are normally colocalised within the endosomes. Roosterman *et al* (2007) identified that ECE-1c however had the largest effect on substance P degradation and NK1R resensitisation.

A similar mechanism could be occurring within the experiment in this study as a result of the over expression of the ECE-1 enzyme. Future work needs to be conducted in order to investigate the other precursor peptides present within human primary NOKs, NOFs and the panel of cell lines in order to determine their expression profiles within the different cell types and the effect that ECE-1 has on them.

There are four different isoforms of ECE-1 and three of the four isoforms, ECE-1b, -1c and -1d are constitutively phosphorylated (MacLeod *et al*, 2002). This phosphorylation event occurs at Ser-18 and Ser-20 and is thought to be conducted by casein kinase I (MacLeod *et al*, 2002). ECE-1a does not contain these residues within its structure (MacLeod *et al*, 2002). Smith *et al*

(2006) determined that the activation of protein kinase C (PKC) by phorbol esters significantly increased the level of ECE-1c phosphorylation. This increase in phosphorylation resulted in an increase of ECE-1 activity at the external surface of the endothelial plasma membrane (Smith *et al*, 2006). Smith *et al* (2006) conclude that this may provide a novel mechanism for the rapid trafficking to and/or activation of ECE-1 at the cell surface which results in an increase in the rate of peptide production. A similar mechanism could be responsible for the increase in HNSCC migration observed after the cells are transfected with the ECE-1c expression vector in this experiment. The continuous phosphorylation of ECE-1c and therefore increase in its activity, could result in the promotion of HNSCC migration.

The treatment of SCC4 cells with an inhibitor to ECE-1 resulted in a small but insignificant decrease in cell migration. Previous studies have identified that the SM-19712 ECE-1 inhibitor can reduce receptor resensitisation triggered by ECE-1 activity (Roosterman *et al*, 2007). The treatment of SCC4 cells in this experiment with this inhibitor did not result in a significant decrease in cell migration. Further experiments need to be conducted in order validate the results. The inhibitor may not be working (a suitable positive control such as a fluorogenic peptide will be required to analyse this; time did not allow this to be tested in this study), or it may be being used at an incorrect concentration and therefore further experiments are required to determine and confirm its activity. In order to specifically reduce the expression of the enzyme SCC4 cells were transfected with siRNA targeted to ECE-1. Transient transfection of SCC4 cells with siRNA to ECE-1 resulted in a decrease in cellular migration of 0.7-fold; however the successful knockdown of the ECE-1 transcript could not be confirmed by qPCR analysis. This result therefore requires further investigation.

7.3 The role of the RAS in promoting head and neck cancer progression via an autocrine mechanism

Studies have shown that local renin-angiotensin system (RAS) components are elevated in a number of diseases including diabetes, cardiovascular disease and diabetic neuropathy (Carey and Siragy, 2003; Goossens *et al*, 2003; Ruiz-Ortega *et al*, 2001). There has been increasing evidence in recent years that the RAS plays an important role in tumourigenesis and cancer progression (George *et al*, 2010). Ang II has mitogenic and angiogenic effects and is the main peptide effector of the RAS. Other components of the RAS include the two GPCRs that Ang II can act through, AT_1R and AT_2R, and the enzymes responsible for the regulatory control of the peptide, angiotensin converting enzyme (ACE) and angiotensin converting enzyme 2 (ACE2). Components of the RAS have been associated with tumour progression and changes in expression levels have been linked to and correlate with tumour grade (Louis *et al*, 2007; Sitzmann *et al*, 1994). The changes however are not always consistent and expression levels of

individual components can vary between tumour types. High AT_1R expression levels are associated with breast hyperplasia however this expression pattern alters as the invasiveness of the tumour increases. Levels of the receptor decrease in this instance (De Paepe *et al*, 2002). In ovarian cancer up-regulation of AT_1R is associated with increased invasiveness (Suganuma *et al*, 2005). The RAS and the ET-axis are both important and potent vasopressor systems (Pollock, 2005). Ang II and ET-1 are both potent vasoconstrictors and for many years researchers have investigated the concerted roles played by both systems in the development of hypertension and the effect they have on each other (Pollock, 2005).

Santos *et al* (2009) identified mRNA for renin, angiotensinogen, ACE, AT_1R and AT_2R in gingival tissue. Renin, angiotensinogen and AT_1R were identified in gingival fibroblasts and renin was detected in the vascular endothelium and intensely detected in the epithelial basal layer of periodontitis affected gingival tissue. The identification of a local RAS in rat gingival tissue suggests that the production of Ang II and other vasoactive peptides within this tissue is possible. The presence of all the essential components shows that a local RAS is capable of generating Ang II in gingival tissue independently of the systemic RAS (Campbell, 1987; Paul *et al*, 2006; Phillips *et al*, 1993 and unpublished data generated in the laboratory).

ACE hydrolyses Ang I at the Phe-8-His-9 bond to generate Ang II. ACE is a zinc-metallopeptidase and is known to be evolutionarily conserved (Macours *et al*, 2004). Two isoforms of ACE exist; somatic ACE and germinal ACE (Lambert *et al*, 2010). Both isoforms of ACE are type-I transmembrane glycoproteins. They consist of an extracellular amino-terminal ectodomain and a short intracellular cytoplasmic tail (Lambert *et al*, 2010). Their structure allows them to hydrolyse peptides, both as a peptidyl dipeptidase (carboxydipeptidase) in the case of Ang I or as an endopeptidase in the case of substance P (Skidgel *et al*, 1984), within the extracellular milieu (Lambert *et al*, 2010). ACE can also hydrolyse BK and N-acetyl-SDKP (Rieger *et al*, 1993). The two homologous catalytic domains in ACE which can also be described as the N- and C- terminals have different substrate and inhibitor profiles (Lambert *et al*, 2010).

Work conducted in this thesis has identified that unlike ET-1, Ang II can significantly stimulate HNSCC migration and invasion via an autocrine mechanism. The treatment of both SCC4 and H357 cells with Ang II significantly stimulated cellular migration. This increase in migration however was not to the same extent as that observed when SCC4 cells were treated with epidermal growth factor (EGF), a potent stimulus for HNSCC migration and invasion. EGF can bind to and activate the EGF receptor (EGFR) resulting in an increase in migration and invasion of SCC10A cells, a HNSCC cell line. The binding of EGF to EGFR resulted in the loss of the

epithelial phenotype associated with SCC10A cells, coupled with downregulation of E-cadherin and the upregulation of both N-cadherin and vimentin. EGF is thought to promote SCC10A cell migration and invasion possibly by an epithelial to mesenchymal transition (EMT)-like phenotype change trigged by its binding to the EGFR (Zuo *et al*, 2011). Treatment of SCC4 and H357 cells with Ang II also resulted in an increase in their ability to invade through Matrigel.

As mentioned previously Ang II can exert its effects by binding to two GPCRs; AT_1R and AT_2R. Ang II exerts most of its pathophysiological effects through the AT_1R. qPCR analysis determined that the AT_1R was over expressed in the primary HNSCCs cell lines and was robustly expressed by NOFs in comparison to NOKs. This result coincided with gene expression profiling databases that identified AT_1R as one of the most prominently over expressed genes in pathophysiological processes (Ager *et al*, 2008). The over expression of AT_1R in primary HNSCC cell lines coincides with the cancer cells ability to be overly sensitive to the mitogenic effects of Ang II. The robust expression of the AT_1R in NOFs can also explain the cell's ability to stimulate HNSCC paracrine migration and invasion after treatment with Ang II. The increased expression of the AT_1R allows the promotion of tumour-stromal interactions between the two cell types, a scenario which could also be present within an *in vivo* environment. An increase in AT_1R expression is associated with numerous cancers including breast and ovarian cancer (Ager *et al*, 2008). The activation of AT_1R by Ang II can result in the stimulation of phospholipase C (PLC)/inositol 1,4,5-trisphosphate/diacylglycerol cascade promoting cellular proliferation, MAPK/ERK tyrosine kinase resulting in stimulation of cell cycle activity and promoting irregular cell growth and rho-associated protein kinase (rho/ROCK) resulting in increased cellular migration (Clempus and Griendling, 2006; Nakashima *et al*, 2006; Saito and Berk, 2002; Touyz, 2005). It can also result in the stimulation of reactive oxygen species (ROS) via a nicotinamide adenine dinucleotide phosphate/nicotinamide adenine dinucleotide phosphate-oxidase (NADP/NADPH) mechanism. The production of ROS can influence downstream signalling molecules including transcription factors, tyrosine kinases/phosphatases, MAPKs and Ca^{2+} channels which promote cell proliferation and irregular cell growth (Clempus and Griendling, 2006; Touyz, 2005). The stimulation of the AT_1R has been implicated in the process of damaged blood vessel revascularization as a result of increased vascular endothelial growth factor (VEGF) production and nitric oxide (NO) synthase stimulated by the binding of the peptide to the receptor (Tamarat *et al*, 2002). This mechanism has also been implicated in ovarian cancer (Suganuma *et al*, 2005).

The use of an AT_1R antagonist confirmed that Ang II was indeed exerting its autocrine effects through this receptor. Telmisartan is commonly used as an anti-hypertensive agent (Ager *et al*, 2008). The use of this receptor antagonist and other AT_1R antagonists including losartan and

candesartan within the clinic identifies possible therapeutic options in the treatment of head and neck cancer. AT_1R therapeutic agents belong to a class of biphenylimidazoles. They can be competitive inhibitors (peptide analogues) or insurmountable receptor antagonists (non-peptide) and exert their effects by restricting the binding of Ang II to the receptor resulting in the prevention of signal transduction (Ager *et al*, 2008).

qPCR analysis determined that the AT_2R was expressed within all primary cells and cell lines but transcript levels of AT_2R seemed to be lower in primary HNSCCs in comparison to NOKs. Evidence has shown that the receptor has been implicated in opposing the effects triggered through the AT_1R and therefore it may be surmised that reduced levels of the receptor would be associated with primary HNSCC cell lines in which an over expression of the AT_1R is apparent, although further studies are required to confirm this at the level of protein expression.

ACE is responsible for the activation of Ang II from the inactive precursor Ang I. The expression of ACE within the same panel of primary cells including primary human NOKs and NOFs and cell lines isolated from primary carcinomas, oral dysplasias and a local metastasis was therefore determined. ACE was expressed within all cell types and was robustly expressed in NOFs in comparison to NOKs. Preliminary work was conducted in order to determine the mechanisms involved in regulating Ang II production and therefore the role of ACE in the production of Ang II was investigated. The over expression of the ACE in SCC4 cells resulted in an increase in HNSCC cellular migration. This result is in keeping with ACE being responsible for the production of Ang II. Other enzymes have been linked to the production of Ang II. The serine-proteinase, chymase has been identified as being capable of generating Ang II from Ang I. Chymase is of great importance in alternative Ang II-generating pathways in several places, including the heart and blood vessels (Cornish *et al*, 1979; Trachte and Lefer, 1979; Schechter *et al*, 1986). Cathepsin A was identified in human renal extracts as being able to generate Ang II from Ang I via the production of an intermediate peptide (Miller *et al*, 1988). There are few reports of expression of either of these enzymes in the oral mucosa, suggesting ACE is likely to be the predominant angiotensinase in this setting. The increase in local Ang II production caused by an increase in ACE activation could result in the peptide stimulating HNSCC cell migration via an autocrine mechanism in a similar manner to that observed when SCC4 were treated directly with Ang II. The results collected in this study suggest that in HNSCC cells, ACE may be responsible for the production of active Ang II, which, coupled with the over-expression of AT_1R identified here, may drive tumour progression Therefore the treatment of head and neck cancer with ACE inhibitors may provide a useful therapeutic option in the treatment of the disease. ACE inhibitors are already used within a clinical practice and are

commonly used in the treatment for hypertension (Ager *et al*, 2008). Studies have also indicated that the incidence of fatal cancers was reduced in patients who had been prescribed ACE inhibitors as a treatment method for longer than three years (Lever *et al*, 1999). Prolonged treatment with the specific ACE inhibitor captopril has been associated with a reduction in the chance of developing prostate cancers (Ronquist *et al*, 2004). Although a number of studies have identified a protective link between ACE inhibitors and the development of cancer, some studies have failed to make this observation and therefore it has been deemed necessary to identify the specific role that the population profiles, the types of cancer examined, the agents used and the dose and length of administration of those agents play in determining the potential benefits associated with ACE inhibitor treatment (Ager *et al*, 2008; Friis *et al*, 2001; Meier *et al*, 2000).

Previous studies have shown that renin and angiotensinogen transcripts are present within gingival tissue (Santos *et al*, 2009). Further investigation needs to be conducted to determine if both renin and angiotensinogen and therefore Ang I are present within HNSCC cell lines, although evidence from other work in the laboratory suggests this is the case. This would further support the fact that ACE over expression within HNSCC cells could result in an increase in Ang II produced by the activity of ACE on locally generated Ang I.

7.4 The role of the ET-axis and the RAS in promoting head and neck cancer progression via a paracrine mechanism

Studies have indicated that ET-1 can stimulate HNSCC proliferation via an autocrine mechanism (Awano *et al*, 2006). This however is not the case for HNSCC migration. Previous reports have discovered that the presence of prostatic stromal fibroblasts can potentiate the effects of ET-1 in prostate cancer (Dawson *et al*, 2004). The tumour microenvironment, also known as the reactive stroma, is composed of a number of cell types with fibroblasts being the most numerous. Fibroblasts found within the stroma surrounding epithelial tumours frequently undergo changes in their phenotype. These changes can include an increase in their migratory nature, increased proliferation and the acquisition of a myofibroblastic and more contractile phenotype. It is thought that these characteristics can be triggered by signals released from the epithelial tumour cells, an example of tumour-stromal interactions. Determining these tumour-stromal interactions could provide interesting and attractive targets for possible future therapeutic strategies and applications.

The treatment of both SCC4 cells and NOFs with ET-1 stimulated HNSCC migration to a greater extent compared to that observed in the absence of NOFs. The inclusion of NOFs into the bottom chamber of the Transwell migration assay created an environment more similar to that

observed *in vivo*. This observation suggests that ET-1 is working via a paracrine mechanism to stimulate the release of soluble factors from the NOFs into the surrounding media which can in turn stimulate HNSCC migration. The seeding of NOFs into the bottom chamber of the Transwell migration assay did not further enhance the effect of BK on HNSCC migration. This thesis has confirmed work conducted by Thomas *et al* (2006) have shown that BK can stimulate HNSCC migration via an autocrine mechanism. This has identified that the direct treatment of HNSCC cells with Ang II results in an increase in their migration and invasive potential. The results observed in this project identify that the peptide can work via an autocrine mechanism similar to that used by BK to also promote HNSCC progression (Thomas *et al*, 2006). The ability of Ang II to activate paracrine stimulation was also investigated.

Treatment with filtered conditioned media collected from ET-1 or Ang II-treated NOFs stimulated the migration of untreated HNSCC cells in a dose responsive manner. This finding suggests that ET-1 and Ang II can both promote the release of factors from NOFs into the surrounding media which are involved in the stimulation of HNSCC cell lines. The presence of the mitogenic peptides alone in the Transwell migration chamber did not increase HNSCC migration suggesting that the presence of NOFs, or the factors that they release, is required to promote HNSCC migration via a paracrine mechanism. The pre-treatment of NOFs with ET-1 or Ang II for 4 h before the media was removed, filtered and added to the bottom well of a Matrigel invasion assay also resulted in the paracrine stimulation of untreated SCC4 and H357 cell invasion suggesting that the peptides are working via a similar mechanism to that observed for HNSCC cell migration. Although Ang II can significantly stimulate HNSCC cell migration and invasion via an autocrine mechanism, the soluble factors released by the NOFs in response to Ang II treatment caused a greater increase in SCC4 and H357 cell migration and invasion in comparison to treatment of the HNSCC cells alone. The ability of ET-1 and Ang II to stimulate HNSCC invasion via a paracrine mechanism is an important finding. The ability of cancer cells to invade through the underlying basement membrane and into the surrounding connective tissue is a vital step in the process of metastasis. Invasion into the surrounding bone and local metastasis to nearby lymph nodes are both common features associated with HNSCC. The development of both processes complicates the treatment of head and neck cancer and ultimately reduces survival rates. Dawson *et al* (2004) have identified that levels of ECE-1 are elevated in the primary malignant prostatic stromal cells in comparison with benign tissue. The specific inhibition of endogenous ECE-1 activity within these stromal cells significantly reduced epithelial cell invasion suggesting that both the enzyme and ET-1 play a role in mediating effects on prostate stroma (Dawson *et al*, 2004). ET-1 has been implicated in the process of bone metastasis that is associated with prostate cancer. It has been

hypothesised that ET-1 can also promote interactions between the prostate metastatic cell and the local environment. The increased production of ET-1 by the prostate cancer cells located in bone is thought to be stimulated by osteoblast- and endothelial cell-secreted IL-1, transforming neurosis factor-α (TNF-α), and tumour growth factor-β (TGF-β). ET-1 can in response stimulate mitotic activity in osteoblasts, decreasing both osteoclastic bone resorption and motility (Nelson *et al*, 1999). The discovery of the unique interactions between the ET-axis, the prostate cancer cells and the bone microenvironment including osteoclasts and osteoblasts, has led to the development of novel therapeutic treatments in prostate cancer (Carducci and Jimeno, 2006). Discovering the role that the ET-axis plays in promoting the invasion of HNSCC and discovering if the axis can contribute to the local metastasis of head and neck cancer into the surrounding bone may help to identify new therapeutic targets in the treatment of the disease.

ET-1 can exert its effects via the ET_AR and/or ET_BR. qPCR analysis conducted in this thesis has identified that the ET_AR is highly expressed within the NOFs and is expressed within the primary carcinoma cell lines; ET_BR transcripts were also present within NOFs suggesting that all the cell types tested have the capacity to respond to ET-1. mRNA levels of the ET_BR were down regulated in the primary carcinoma-derived cell lines which is in keeping with previous reports (Dupuis *et al*; 2000). This result however contradicts the findings of Awano *et al* (2006) who observe an increase in ET_BR transcript levels in HNSCCs. However, in that study skin keratinocytes were used as the 'normal' control and this may explain the differences in ET_BR expression observed. The ability of antagonists to both receptor subtypes, to block the effects observed in this thesis suggests that ET-1 uses both receptors in the NOFs to stimulate HNSCC cell migration and invasion.

Ang II can exert its effects through both the AT_1R and AT_2R. The peptide exerts most of its pathophysiological effects through AT_1R. qPCR analysis conducted within this project identified that the highest expression of the AT_1R, across the panel of primary cells and cell lines tested, was in NOFs, suggesting that it has an important role within these cells. The ability of Ang II to act through this receptor was supported by the finding that telmisartan, a specific AT_1R antagonist, was able to block the paracrine stimulation of SCC4 cells by NOF treatment with Ang II. The extent of AT_1R expression in NOFs and HNSCC primary carcinoma cell lines may mean that the receptor could be a possible therapeutic target in the treatment of HNSCC. Studies have shown that AT_1R can be regulated at a post-transcriptional level (Nickenig and Harrison, 2002) via mechanisms including Ang II (Nickenig and Murphy, 1996), estrogens (Krishnamurthi *et al*, 1999) and cyclic adenosine monophosphate (cAMP) stimulating agents (Wang *et al*, 1997) which all decrease rat mRNA levels of the receptor by targeting it for

degradation. Insulin (Nickenig *et al*, 1998), progesterone (Nickenig *et al*, 2000) and low density lipoproteins (Nickenig *et al*, 1997) all act in the opposite manner and increase AT_1R mRNA levels by decreasing the rate at which the mRNA is decayed. Cytosolic proteins can also regulate AT_1R expression at a post-transcriptional level by binding to 5'-untranslated region (UTR) region of the AT_1R mRNA (Krishnamurthi *et al*, 1999; Lee *et al*, 2006; Ji *et al*, 2004; Zhang *et al*, 2004; Wu *et al*, 2003; Mok *et al*, 2003). The complex regulatory system involved in Ang II signalling, which has been identified here, must be taken into account when considering the AT_1R as a therapeutic target in the treatment of cancers. The mechanisms responsible for its upregulation in HNSCC cells remain to be determined.

The results presented in this thesis suggest paracrine stimulation of HNSCC migration and invasion is the result of the ET-1 or Ang II-stimulated release of bioactive ligands from the surface of the NOFs. The mechanisms involved in this stimulation were further elucidated. The ADAMs family of enzymes are known to promote signalling and play a key role in the control of the processes that occur and exist between different cells and between cells and their microenvironment (Murphy, 2008). About half of the ADAMs family are known to have proteolytic activity and can modulate the activities of membrane growth factors and cytokines and their receptors and cell adhesion molecules (Murphy, 2008). The ability of ADAMs enzymes to affect and alter these processes implicates them in the promotion and progression of tumourigenesis (Murphy, 2008). A number of ADAMs enzymes are upregulated or their activity increased in different cancers (Rocks *et al,* 2008; Mochizuki and Okada, 2007). The involvement of ADAMs enzymes in the release of factors from the surface of cells therefore warranted further investigation in order to determine if the enzymes were involved in the paracrine mechanism stimulated by ET-1 and Ang II treatment of NOFs. In order to conduct this investigation NOFs were pre-treated with a broad range inhibitor to both the ADAMs enzymes and MMPs, GM6001. NOFs were pre-treated with this inhibitor for 30 min before the addition of ET-1 or Ang II and this resulted in the inhibition of ET-1 and Ang II stimulated HNSCC cellular migration. This observation suggests that a member of the ADAMs family of enzymes or a MMP could be involved in the process. ADAM17 is the member of the family most commonly responsible for the proteolytic release of soluble factors from the surface of cells (Murphy, 2008). siRNA targeted to ADAM17 was used to knock down its expression within the NOFs in order to determine its effect HNSCC cell migration. The successful transient transfection of NOFs with siRNA to ADAM17 resulted in the inhibition of ET-1 and Ang II stimulated SCC4 migration. qPCR analysis confirmed that the siRNA was only targeting ADAM17 and not other members of the family including ADAMs -9, -10 and -15, suggesting

that ADAM17 is at least in part involved in the paracrine mechanism stimulated by ET-1 and Ang II.

EGFR signalling is a pathway commonly deregulated in a number of cancers. The EGFR receptor is a receptor tyrosine kinase. Activation of the receptor via ligand induced autophosphorylation, can lead to downstream signalling including that of the mitogenic Ras/Raf/ERK 1/2, p38 MAPK and PI3-K/AKT pathways, all of which are involved in regulating cell proliferation, growth and differentiation (Cai *et al*, 2010; Ciardiello and Tortora, 2008; Avraham and Yarden, 2011). Aberrant activation of the EGFR can also promote an increase in cell migration and invasion in HNSCC (Thomas *et al*, 2006).

The EGFR can also be transactivated via GPCRs. A number of GPCR ligands have been identified as being able to activate the EGFR, including BK in HNSCC (Thomas *et al*, 2006). This GPCR-mediated EGFR activation has also been apparent in prostate and breast cancer cell lines (Filardo *et al*, 2000; Prenzel *et al*, 1999). Lui *et al* (2003) discovered that the gastrin-releasing peptide (GRP) receptor could activate EGFR resulting in the alteration and promotion of HNSCC growth and invasion. The EGFR has also been activated in HNSCC cases in response to lysophosphatidic acid (LPA) and thrombin ligands (Gschwind *et al*, 2003). The GPCR-mediated EGFR activation results in the triggering of downstream signalling pathways including p44/42 MAPK (Gschwind *et al*, 2001; Santiskulvong *et al*, 2001). Depending on the cell type and the ligand present the activation of MAPK can be as a result of EGFR-dependent and/or EGFR-independent mechanisms (Thomas *et al*, 2006). BK in vulvar carcinoma A431 cells activated MAPK via an EGFR-independent mechanism which involved PI3-K and PKC pathway (Graness *et al*, 2000). Other intracellular pathways that have been implicated in GPCR-mediated EGFR activation include serine/threonine kinase PKC (Slack, 2000), increased Ca^{2+} levels (Zwick et al, 1997) and the nonreceptor tyrosines kinases from the Src family (Luttrell et al, 1997). Prenzel *et al* (1999) have suggested that the extracellular domain of EGFR is involved in the cross talk that occurs between the receptor and the GPCR. In bladder and kidney cancer cells the ADAMs -10, -15 and -17 have been implicated in the activation of EGFR proligands (Schafer *et al*, 2004). In HNSCC ADAM17 is in involved in GPCR-mediated EGFR activation and inhibition of the enzyme has resulted in a reduction in PGE_2-stimulated and BK-stimulated downstream signalling events (Gschwind *et al*, 2003; Zhang *et al*, 2006; Schafer *et al*, 2004). In the study presented in this thesis it was found that the pre-treatment of HNSCC with an EGFR antagonist before their addition to the Transwell migration assay resulted in the inhibition of SCC4 cell migration in the presence of ET-1 or Ang II treated NOF conditioned media. This suggests that the release of soluble factors from the surface of the NOFs in response to ET-1 or Ang II treatment can act as ligands for the EGFR located on the surface of the SCC4 cells resulting in

its activation and an increase in the migration and invasion of the epithelial cells. Pre-treating NOFs for 30 min with receptor inhibitors to both ET_AR and ET_BR before the stimulation with ET-1 and SCC4 cells with an EGFR receptor antagonist before their addition to the Transwell migration assay resulted in the further inhibition of HNSCC migration. The incubation of conditioned media collected from NOFs treated with ET-1 with neutralising antibodies to the EGFR ligands transforming growth factor-α (TGF-α), heparin bound (HB)-EGF and amphiregulin blocked stimulation of SCC4 migration. Both results further implicate EGFR transactivation in the paracrine mechanism involved in the stimulation of HNSCC cell migration and invasion. These findings add weight to the suggestion that the combined inhibition of both GPCR and EGFR could lead to enhanced antitumour effects in comparison to the treatment of other the GPCR or EGFR independently (Thomas *et al*, 2006), and give the first indication that this approach may be indicated in HNSCC.

qPCR analysis identified that the activation of the EGFR by ectodomains released from the surface of NOFs in response to ET-1 treatment, also stimulated an increase in the expression of cyclooxygenase-2 (COX-2). COX-2 and EGFR are both frequently over-expressed in HNSCC and correlate with a poor prognosis (Chan *et al*, 1999; Kalyankrishna and Grandis, 2006). COX-2 is known to play an important role in promoting HNSCC migration by increasing the levels of PGE_2 (Wang et al, 2005). PGE_2 is a pleiotropic factor which can stimulate COX-2 expression and promote cancer cell motility (Wang *et al*, 2005). PGE_2 has also been shown to transactivate the EGFR (Kalyankrishna and Grandis, 2006) suggesting that this may form a positive feedback loop in response to factors released from the surrounding stroma in the presence of ET-1. Segawa *et al* (2003) discovered that Ang II treatment of human gingival fibroblasts can also result in an increase in PGE_2 release. Other mediators of inflammation including BK, histamine, IL-1β and TNF-α are also known to increase PGE_2 production in human gingival fibroblasts (Lerner and Modéer, 1991; Modéer *et al*, 1993; Yokota *et al*, 1994; Niisato *et al*, 1996; Nakao *et al*, 2000). An increase in intracellular Ca^{2+} after treatment was observed, suggesting that AT_1R activation by Ang II stimulates this increase (Segawa *et al*, 2003). This evidence, along with that presented in this thesis, suggests a possible paracrine mechanism via which mitogenic peptides including ET-1 can promote HNSCC migration and invasion. It is plausible to suggest that the activation of the EGFR via Ang II treatment could result in an increase in COX-2 expression, similar to that observed for ET-1 paracrine stimulated SCC4 migration and invasion. This remains to be determined but evidence collected from other studies have also identified a role that Ang II plays in stimulating COX-2 expression in lung fibroblasts (Matsuzuka *et al*, 2009). This evidence and that collected in this study using the ET-1 mitogenic peptide indicates that Ang II stimulation of NOFs may also result in an increase in

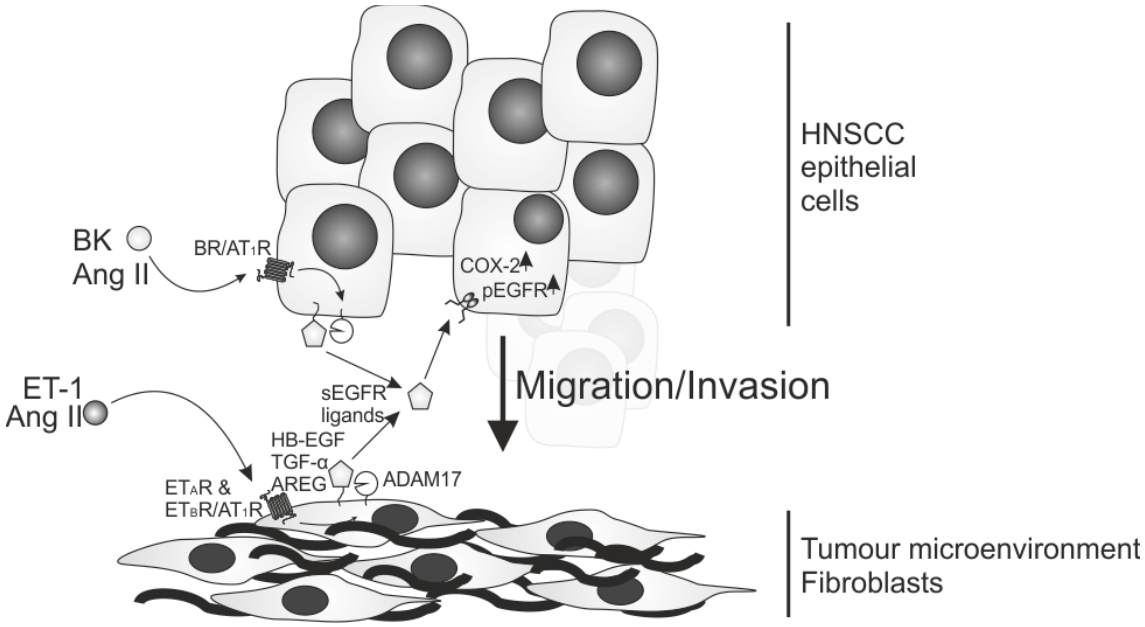


Figure 7.1 Schematic to illustrate how ET-1, Ang II and other mitogenic peptides may promote HNSCC cell migration and invasion either by promoting stromal-tumour interactions (ET-1 and Ang II) or by acting directly on tumour epithelial cells (Ang II and BK): The mitogenic peptides ET-1 and Ang II bind to their specific GPCRs, ET_AR and ET_BR for ET-1 and AT_1R for Ang II, which are located on the NOF cells. This binding event results in the activation of the ADAMs proteinase enzyme, ADAM17. ADAM17 is a member of the ADAMs family that is commonly associated with and responsible for triggering the release of soluble factors including HB-EGF, TGF-α and amphiregulin from the surface of NOFs which can act as ligands for the EGFR located on the surface of the HNSCC epithelial cells. The binding of these ligands to the EGFR results in its activation and an increase in the migration and invasion of the epithelial cell on which the receptor is located. A broad range inhibitor to the ADAMs family of enzymes, siRNA to ADAM17 and an antagonist to the EGFR result in the inhibition of HNSCC cell migration. The activation of the EGFR coincides with an increase in COX-2 expression, suggesting a possible intracellular mechanism responsible for the increase observed in HNSCC migration. Ang II, like BK, can also stimulate HNSCC migration and invasion via an autocrine mechanism in which the mitogenic peptides bind directly to AT_1R on the HNSCC epithelial cells.

COX-2 expression in the HNSCC microenvironment, possibly contributing to cancer progression (Figure 7.1).

HNSCCs are highly inflammatory and aggressive cancers in nature and express a number of growth factors and cytokines which are involved in the inflammatory process (Wang *et al*, 2009). The risk factors associated with head and neck cancer including the consumption of both alcohol and tobacco and exposure to the human papillomavirus (HPV) have all been linked to increased pro-inflammatory cytokine expression and the induction of inflammatory signalling pathways including nuclear factor-kappa B (NF-κB), JAK/STAT and PI3-K/Akt/mTOR (Wang *et al*, 2009), thereby implicating inflammation in head and neck cancer progression. Increased NF-κB signalling is associated with an increase in COX-2, a target gene of NF-κB (Wang *et al*, 2009). As mentioned previously COX-2 is associated with poor prognosis in HNSCC and the increase in the expression observed in this thesis suggests that the inflammatory response and subsequent signalling activation could be responsible for this increase in expression. Further work needs to be conducted in order to determine the signalling mechanisms activated by factors found within the tumour microenvironment.

ET_AR, ET_BR and AT_1R antagonists and EGFR and COX-2 inhibitors are currently being utilised in clinical trials for the treatment of other malignancies (Nelson *et al*, 2003; Ager *et al*, 2008). The involvement of these different components of the ET-axis and RAS have been implicated, as a result of the work conducted in this thesis, in mechanisms involved in head and neck cancer progression. Therefore the targeting of the different factors and the implementation of combined therapy may have considerable significance in the treatment of HNSCC and therefore warrants further investigation.

microRNAs (miRNAs) are small regulatory RNAs recently identified as playing a major role in the control of expression of genes that are involved in cancer progression. It has been suggested that they can play an important part in regulating cellular processes including migration, invasion and metastasis. ADAM17 has been identified as being a target of miR-145 (unpublished data generated in the laboratory), a putative tumour suppressor miRNA down-regulated in HNSCC. The over-expression of miR-145 in HNSCC cells reduces their ability to migrate in the presence of various stimuli (unpublished data generated in the laboratory). These two findings and the identification of the important role ADAM17 plays within the paracrine stimulation of HNSCC within this study made it logical to investigate the role of miR-145 in the paracrine stimulation of HNSCC migration activated by ET-1 and Ang II treatment. Heterologous expression of miR-145 in NOFs resulted in the inhibition of conditioned media-treated SCC4 migration in both the presence and absence of ET-1 and in the presence of Ang II.

This suggests that the miRNA can exert its effects by targeting the expression of ADAM17 and as a result influencing the activities that are controlled and activated by ADAM17 itself (although it is likely to have other contributory targets also). This includes affecting the release of factors from the surface of NOFs both in response to ET-1 treatment and basal ligand shedding in its absence. This finding further suggests that ADAM17 is involved in regulating interactions within a microenvironment in a normal cellular situation and that alteration within its expression, a characteristic observed in a number of malignancies, could result in the uncontrolled behaviour of this microenvironment. miR-145 is a possible feature within this microenvironment that could be responsible for the deregulation of ADAM17 and could arguably be considered as a possible drug target for the treatment of HNSCC. The transfection of NOFs with miR-145 resulted in a greater inhibition of SCC4 migration in comparison to NOFs transfected with ADAM17 siRNA. This finding suggests that miR-145 exerts its effects by inhibiting more than one factor, whereas transfection of NOFs with siRNA to ADAM17 results in the specific targeting of ADAM17 only. miR-145 has been identified as being down regulated in cancer associated fibroblasts (CAFs) isolated from a carcinoma of the bladder (Enkelmann *et al*, 2011), further fuelling this hypothesis. The other targets of miR-145 and the role that they play in the tumour microenvironment need to be investigated in order to determine the full potential of the miRNA as a possible treatment of cancers including head and neck.

Other links have been made between miRNAs and fibroblasts found within the tumour microenvironment, further supporting the evidence and results collected in this thesis. Musumeci *et al* (2011) identified that miR-15 and miR-16 were both down regulated in fibroblasts surrounding prostate tumours. The down regulation that was observed resulted in increased tumour growth and progression (Musumeci *et al*, 2011). The group demonstrated that this promotion of tumourigenesis was due to the reduced post-transcriptional repression of fibroblast growth factor (FGF)-2 and its receptor, FGF receptor (FGFR)-1. Studies have shown that FGF-2 production by both tumour and stromal cells leads to increased proliferation and metastasis formation in prostate cancer (Cronauer *et al*, 1997; Yang *et al*, 2008; Giri *et al*, 1999). Transfection of miR-15 and miR-16 into prostate cells resulted in a decrease in the tumour-supportive capability of stromal cells both *in vivo* and *in vitro* (Musumeci *et al*, 2011). This observation and the evidence collected in this study identify a role for miRNAs in tumour-stromal interactions and suggest that miRNAs in the stroma surrounding epithelial tumours could provide alternative therapeutic targets in the treatment of cancer.

Li *et al* (2010) have also identified that endothelial miR-125a/b-5p can inhibit ET-1 expression in vascular endothelial cells therefore suggesting that individual components of the ET-axis

could be regulated by miRNAs. miR-125a/b-5p target the 3'-UTR of pre-pro ET-1 mRNA in order to achieve this inhibition. Additional research has also identified the involvement of miR-155 in the control the RAS. Zheng *et al* (2010) have identified that AT_1R is a target of miR-155 and have shown that transfection of adventitial fibroblasts with miR-155 resulted in a decrease in AT_1R protein levels but not mRNA levels. A reduction in ERK 1/2 signalling and a reduction in stimulation of α-SMA) by Ang II treatment was also observed after transfection with miR-155 (Zheng *et al*, 2010). The results suggest that the miRNA could play a role in regulating adventitial fibroblast transdifferentiation via the AT_1R (Zheng *et al*, 2010). Martin *et al* (2006) show that miR-155 can interact with the 3'-UTR region of AT_1R. Transfection of the miRNA into human primary lung fibroblasts led to a reduction in AT_1R mRNA and a reduction in ERK 1/2 activation triggered by Ang II stimulation (Martin *et al*, 2006). When the fibroblasts were treated with TGF-β the level of miR-155 was reduced and mRNA levels of AT_1R were increased (Martin *et al*, 2006). This evidence and that collected within this study support the fact that miRNAs may play a role in controlling the ET-axis and RAS and may be responsible for alterations observed within the systems that are associated with patho-physiological scenarios.

As mentioned previously, ECE-1 is responsible for the biological activation of ET-1. This study has identified that the over expression of the enzyme in HNSCC resulted in an increase in cell migration. The over expression of the enzyme in SCC4 cells and the increase in cellular migration that was observed as a result was not thought be have been as a result of increased production of biologically active ET-1. SCC4 cells did not respond to the autocrine activation stimulated by ET-1 treatment only therefore it was not logical to suggest that the over expression of ECE-1 promoted SCC4 cell migration as a result of the increased production of the peptide. HNSCC cells however did respond to the paracrine stimulation triggered by the treatment of NOFs with ET-1. It is therefore sensible to suggest that expression of the enzyme in NOFs may result in the production of biologically active ET-1 which can stimulate an increase in SCC4 migration via a similar paracrine mechanism to that observed with direct treatment of the NOFs with the mitogenic peptide. ECE-1 is also responsible for the hydrolysis of other biologically active peptides including BK, neurotensin and substance P which could result in an increase in HNSCC migration via a similar mechanism to that observed for the autocrine stimulation of HNSCC cells which may have involved prolonged intracellular signalling triggered by the mitogenic peptides in response to the internalisation and recycling of their GPCRs to the plasma membrane activated by ECE-1 activity. Work is currently ongoing to assess any changes in ECE-1 expression and ET-1 production in cancer associated fibroblasts from HNSCC patients.

The treatment of NOFs with a broad range inhibitor to ECE-1 for 30 min before the addition of ET-1 resulted in a small decrease in SCC4 cell migration stimulated with condition medium from the NOFs. This was however not to a significant extent suggesting that other factors or enzymes aside from ET-1 and ECE-1 are contributing to the paracrine stimulation of HNSCC. This is a similar result to that observed when SCC4 cells were treated directly with the ECE-1 inhibitor. The small reduction observed in this case may again need further experiments to be conducted in order to understand the mechanisms underlying this observation and top determine if the inhibitor is working correctly. The direct targeting and reduction in ECE-1 activity using siRNA to the enzyme however did result in a significant reduction in SCC4 migration. This could not be confirmed however by qPCR analysis and therefore warrants further investigation. The result does suggest that ECE-1 is important in the paracrine stimulation of HNSCC migration and invasion and the inactivation of the enzyme may result in a reduction in the activity and production of biologically active peptides including ET-1, which are responsible for paracrine stimulation of HNSCC migration.

When HNSCC cells were transiently transfected with a pIRES vector containing the coding region for somatic ACE, an increase in cellular migration was observed. Over expression of ACE in SCC4 cells, confirmed by qPCR analysis, is likely to cause an increase in the production of biologically active Ang II which in this study has been shown to be able to stimulate the migration and invasion of HNSCCs via an autocrine mechanism. This experiment identified a role for ACE in the autocrine signalling mechanism stimulated by Ang II treatment. A role for the enzyme in the paracrine signalling mechanism stimulated by the peptide was investigated next. In order to investigate the effect of over expressing the enzyme in NOFs and determining the effect on the paracrine stimulation of HNSCCs, NOFs were transiently transfected with the same vector containing the coding region of somatic ACE. The successful transfection of NOFs with ACE, which was confirmed by qPCR analysis, resulted in a significant increase in the paracrine stimulation of SCC4 cell migration. This result suggests that ACE over-expression in NOFs could produce biologically active Ang II which can then go on to stimulate the SCC4 cells via a paracrine mechanism similar to that observed when NOFs were treated with exogenous Ang II.

7.5 The effect of ET-1 and Ang II of the phenotype of NOFs

In a number of malignancies it has been shown that cancerous cells can increase the levels of numerous factors in the reactive stroma that surrounds them that have the ability to activate fibroblasts, resulting in their conversion to a myofibroblast or protomyofibroblast phenotype. Myofibroblasts have been identified in the microenvironment that surrounds a number of different tumours including invasive breast cancers and HNSCCs (Sappino *et al*, 1988).

Myofibroblasts are characterised by high expression levels of α-SMA and MMP-2 and increased cell proliferation, migration and cellular contraction (Allinen *et al*, 2004).

Here, the effect of ET-1 on the proliferation of NOFs was determined using an MTS assay. ET-1 significantly stimulated NOFs to proliferate after 48 h. The effect of the peptide was blocked using receptor antagonists to ET_AR and ET_BR further confirming that the peptide is acting through both receptors in NOF (as previously shown; Section 4.4)

Fibroblasts, when activated, can become more migratory. In order to determine the effect of ET-1 and Ang II on NOF migration, a 2D migration assay was used. The creation of a 'scratch' within a confluent monolayer of NOFs and their subsequent treatment with ET-1 or Ang II resulted in significantly more migration into the 'scratch' than that observed when NOFs were treated with serum free media. This effect, stimulated by ET-1 (in the presence of mitomycin C to negate the contribution of the increased proliferation), could again be inhibited by the use of receptor antagonists to ET_AR and ET_BR. ET-1 is known to promote an increase in both migration and proliferation of rabbit, guinea pig and rat gingival fibroblasts (Ohsawa *et al*, 2005; Ohuchi *et al*, 2002; Ohuchi *et al*, 2010). In another instance Yahata *et al* (2006) showed that AT_1R knockout mice experience delayed wound healing and identified that the treatment of fibroblasts and keratinocytes with Ang II resulted in an increase in their migration (Yahata *et al*, 2006). The process of wound healing epithelialisation and dermal repair require an increase in the migration, proliferation and differentiation of keratinocytes and an increase in ECM production by fibroblasts, respectively (Hashimoto, 2000). Inhibition of AT_1R and an antagonist to HB-EGF resulted in a decrease in fibroblast and keratinocyte migration suggesting that both are involved in the mechanism and the wound healing process triggered by Ang II (Yahata *et al*, 2006). Haddow (1972) first identified and documented similarities between carcinogenesis and wound healing. In 1986, Dvorak *et al* described cancer as 'wounds that do not heal'. The molecular mechanisms involved in the wound healing process therefore provide valuable evidence and could help to create possible hypotheses that can be used in cancer research. The discovery of the ability of ET-1 and Ang II to promote NOF migration, and work conducted previously which has identified that the RAS is involved in the wound healing process, further implicate both the ET-axis and RAS in the activation of fibroblasts and the creation of a unique tumour microenvironmental niche.

ET-1 has been shown to exhibit mitogenic activity towards a number of cells types including fibroblasts and smooth muscle cells (Levin, 1995) and also promotes fibroblast contraction (Guidry and Hook, 1991). The contraction of fibroblasts is an important action in the processes of wound healing and dermis reconstitution (Grinnel, 1994). ET-1 is also capable of modifying

the ECM (Levin, 1995), increasing collagen I and III synthesis, and decreasing the mRNA and protein levels of MMP-1 in dermal fibroblasts (Xu *et al*, 1998; Shi-Wen *et al*, 2001), all processes which are involved in wound healing. MMPs are a group of enzymes that have been implicated in the remodelling and destruction of the ECM, a process which is critical in tumour progression (Coussens *et al*, 2002). The destruction of the basement membrane by MMPs allows tumour cells to invade into the surrounding connective tissue, entry and exit into and out of blood vessels and therefore aid metastasis to distant locations (Coussens *et al*, 2002). MMPs are known to be up-regulated in many cancers (Egeblad and Werb, 2002) and have therefore been extensively investigated as possible therapeutic targets in the treatment of cancer (Coussens *et al*, 2002). The treatment of fibroblast:collagen lattices with both ET-1 and Ang II resulted in the stimulation of their contraction suggesting that the peptides can promote a more contractile phenotype within NOFs. In this investigation on the influence of the peptide on the phenotype of NOFs, the ET_BR seems to be predominantly responsible for the actions of ET-1 and its ability to stimulate the increased contractile nature of the cells. A similar observation was noted by Knowles *et al* (2012) who identified that the inhibition of the ET_AR alone was sufficient to abrogate the effect of ET-1 on the growth and proliferation of colonic fibroblasts but that the blocking of ET_BR with a specific inhibitor was required in order to block the effect of ET-1 on colonic fibroblast contraction. In this regard ET-1 could be using similar mechanisms to that observed in lung fibroblasts; Shi-Wen *et al* (2004) who observed that the treatment of normal lung fibroblasts with ET-1 can result in the cells exhibiting a more contractile phenotype and caused by an increase in the expression of α-SMA, paxillin, moesin and erzin. This process was inhibited by blocking the PI3-K/AKT signalling pathway (Shi-Wen *et al*, 2004). ET-1 exerts its affects by binding to the ET_AR on normal lung fibroblasts and can increase collagen matrix contraction and the production of α-SMA stress fibres (Shi-Wen *et al*, 2004). Secretion of the peptide is associated with fibroblasts isolated from the scars of patients suffering with sclerodema (Shi-Wen *et al*, 2004).

The source of myofibroblasts within the tumour environment remains controversial but there is strong evidence to support the conversion of normal fibroblast to a more 'activated' form; the results shown here suggest ET-1 and Ang II may play a role in this. Studies have identified that TGF-β and ET-1 can promote myofibroblast transdifferentiation (Desmouliere *et al*, 1993; Leask, 2008; Leask, 2010) and Ang II can stimulate myofibroblast transdifferentiation in lung fibrosis (Marshal *et al*, 2004). Myofibroblasts can also produce more ET-1 in comparison to normal fibroblasts suggesting that this may contribute to the higher levels of the mitogenic peptide commonly found within the tumour microenvironment. Work is ongoing in the laboratory to determine whether activated myofibroblast-derived ET-1 may contribute to

HNSCC pathogenesis. Human macrophages (Ehrenreich *et al*, 1990) and human monocyte-derived dendritic cells (Spirig *et al*, 2009), both of which are cell associated with the inflammatory cascade, can also produce ET-1. Alvarez *et al* (2011) identified that ET-1 was produced and released from macrophages activated by Fcγ receptor (FcγR)/toll-like receptor (TLR)-7. The production of increased levels of ET-1 resulted in its ability to act as a profibrotic factor in the inflammatory signalling cascade and leading to the resident cardiac fibroblasts releasing TGF-β and promoting their transdifferentiation and scarring of the within the local area (Alvarez *et al,* 2011). It is noteworthy in this context that inflammation is associated with poor prognosis in HNSCC (Wang *et al*, 2010); any contribution of ET-1 to this remains to be determined. Alvarez *et al* (2011) recently showed an increase in collagen synthesis and α-SMA was observed in the cardiac fibroblasts treated with ET-1. Antagonists to both ET_AR and ET_BR resulted in a decrease in this activity suggesting that ET-1 is working via both receptors in the fibroblasts. This finding supports work conducted in this study which has identified that ET-1 is stimulating paracrine stimulation of HNSCC migration and invasion via both the ET_AR and the ET_BR. The expression of both receptors varies greatly between tissue and species type and it has been suggested that their expression could change depending on developmental stage, growth and health conditions (Hafizi *et al*, 2004).

TGF-β is a multifunctional cytokine and is a well characterised stimulant of myofibroblast transdifferentiation. Treatment of NOFs with TGF-β resulted in a significant increase in both MMP-2 and α-SMA mRNA levels and α-SMA protein levels. MMP-2 and α-SMA are both used as markers to determine the existence of myofibroblasts (Sobral *et al*, 2011). In contrast, treatment of the cells with ET-1 resulted in a small but not significant increase in the mRNA of MMP-2 and α-SMA and protein levels of α-SMA within the NOFs. This result suggests that the peptide is having some effect on the cells but that they are not fully differentiating into myofibroblasts as a result of treatment with ET-1. A number of different types of activated fibroblasts have been found and identified within the tumour microenvironment and these fibroblasts have been described as having a broad spectrum of morphological phenotypic entities. These phenotypic characteristics can range from the non-contractile fibroblast to the contractile myofibroblast which expresses α-SMA, with a number of intermediate phenotypes having been described in between the two extremes (Eyden, 2005) which are often described as protomyofibroblasts (Desmouliere *et al*, 2003). Although treatment of NOFs with ET-1 does not stimulate an increase in α-SMA and MMP-2 expression in comparison to untreated NOFs, the mitogenic peptide is altering the phenotypic characteristic of the cells; increasing their proliferative and migrational ability and making them more contractile. This suggests that ET-1

treatment of NOFs results in a phenotypic change associated with the intermediate spectrum of fibroblast activation.

The treatment of NOFs with Ang II for 48 h resulted in a significant increase in the mRNA levels of both MMP-2 and α-SMA. This stimulation however was not to the same extent as that observed with TGF-β treatment further implementing the broad spectrum of phenotypic characteristics associated with activated fibroblasts found within the reactive stroma. The increase in α-SMA transcript levels activated by Ang II treatment could be reduced by the pre-treatment of NOFs with telmisartan suggesting that Ang II is stimulating the transdifferentiation of the NOFs into myofibroblasts via the AT_1R.

Previous work demonstrates that ET-1, Ang II and TGF-β can interact with each other to alter the behaviour of cells and the regulatory systems responsible for the control of the individual peptides (Lagares *et al*, 2010; Shi-Wen *et al*, 2007; Shephard *et al*, 2004; Alvarez *et al*, 2011). A TGF-β-ET-1 axis has been suggested in the fibrotic process. TGF-β can induce ET-1 expression in human dermal fibroblasts. Within this scenario the TGF-β1 induced expression of profibrotic genes was dependent on ET-1 (Lagares *et al*, 2010). This finding suggests that the activity and control of ET-1 and TGF-β is dependent on each other in this example. ET-1 can mediate the induction of profibrotic genes including CCN2, type I collagen and fibronectin in human lung fibroblasts in the presence of TGF-β (Shi-Wen *et al*, 2007). Shephard *et al* (2004) suggest that TGF-β can act in an autocrine manner to amplify the production of ET-1. This evidence supports work conducted by Alvarez *et al* (2011) who suggested that fetal cardiac fibroblasts may secret TGF-β in response to ET-1 which is secreted by macrophages infiltrating the area which could lead to further production of ET-1.

It was therefore deemed necessary to begin to investigate the role that TGF-β might play in the mechanisms triggered by ET-1 and Ang II stimulation. Previous studies conducted have helped to support the rational for this next series of experiments and have also identified that both the ET-axis and RAS can affect the expression and function of the cytokine itself. Ang II treatment of human lung fibroblasts resulted in an increase in TGF-β1 synthesis (Marshall *et al*, 2004; Marshall *et al*, 2006). Martin *et al* (2007) identified that TGF-β1 stimulation results in the activation of PI3-K, p38K and JNK signalling pathways and phosphorylation of Smad proteins at additional serine threonine sites resulting in their translocation to the nucleus with Smad4 and an increase in AT_1R expression, an example of a TGF-β responsive gene. The crosstalk between the pathways and Smad complex are crucial in the ability of TGF-β to modulate AT_1R expression (Martin *et al*, 2007). TGF-β1 phosphorylates Smad2 and -3 within their conserved COOH-terminal SSXS motif (Derynk and Zhang, 2003; Shi and Massague,

2003). The activation of these signalling pathways could also result in direct and indirect phosphorylation of transcription factors which in turn translocate to the nucleus and merge their signal with the activated Smad complex again resulting in an increase in AT_1R expression (Martin *et al*, 2007). The results suggest that a self-potentiating loop may exist between TGF-β and Ang II and both may be involved in pulmonary fibrosis (Martin *et al*, 2007).

Altered TGF-β signalling has been associated with a number of diseases (Konigshoff *et al*, 2009). In chronic obstructive pulmonary disease, increased TGF-β signalling is associated with increased ECM within patient's distal airways (Neptune *et al*, 2003). Reduced TGF-β signalling is also a factor present within the disease and can be associated with suboptimal matrix deposition which may result in reduced repair of the airspace compartment within the lung which can lead to histologic emphysema (Habashi *et al*, 2006). Within the kidney and the myocardium, enhanced renin-angiotensin-aldosterone signalling is associated with fibrosis (Kagami *et al*, 1994; Zhou *et al*, 2006). Within these examples angiotensin promotes increased TGF-β expression and signalling which can promote EMT transition and epithelial cell apoptosis within the lung resulting in its injury (Kagami *et al*, 1994; Zhou *et al*, 2006). Angiotensin receptor inhibitors can attenuate this effect (Li *et al*, 2003; Marshall *et al*, 2004). Podowski *et al* (2012) identified that the use of losartan, an AT_1R inhibitor used clinically to antagonise TGF-β signalling resulted in improved oxidative stress, elastin remodelling, metalloprotease activation and inflammation within the lungs of mice suffering with chronic obstructive pulmonary disease.

Burns *et al* (2010) identified that Ang II can stimulate TGF-β1 synthesis and EMT. Zhou *et al* (2010) showed that the activation of the AT_1R was associated with glucose stimulated EMT. The effects triggered through the receptor could be reduced but not completely blocked using antagonists to the receptor. In 2012, Zhou *et al* showed that high glucose concentration within renal epithelial cell lines resulted in decreased mRNA levels of the Mas receptor and ACE2. This was coupled with an increase in α-SMA and vimentin mRNA levels, an increase in TGF-β1 and fibronectin synthesis and production and a decrease in E-cadherin which is normally associated with EMT. Treatment with Ang 1-7 resulted in a decrease in levels of α-SMA, vimentin, TGF-β1 and fibronectin and increased levels of E-cadherin. Ang 1-7 treatment also resulted in a decrease in ERK and p38 phosphorylation which had previously been stimulated by the high glucose treatment. The peptide however did not alter the increased stimulation of JNK phosphorylation stimulated by high glucose (Zhou *et al*, 2012).

In light of all this evidence, the synergistic effect of combining ET-1, Ang II and/or TGF-β on NOFs and on the paracrine stimulation of HNSCC migration was investigated using a Transwell

migration assay. The synergistic effect of all three peptides has been implicated in the process of myofibroblasts transdifferentiation in cardiac fibroblasts. The combined treatment of NOFs with ET-1 and Ang II and ET-1 and TGF-β resulted in the further increase in SCC4 migration in comparison to that observed when the NOFs were treated with the peptides individually. These effects however were not significant. The treatment of NOFs with Ang II and TGF-β and ET-1, Ang II and TGF-β however did result in a significant further increase in SCC4 migration in comparison to that observed when NOFs were treated with each peptide alone. The combined treatment of NOFs with ET-1, Ang II and/or TGF-β resulted in further paracrine stimulation of SCC4 cells in comparison to treatment of NOFs with the peptides individually. Although these results were not always significant there was a definite increase in the overall trend. Further experiments are required to form a conclusive result however it is clear that the paracrine stimulation of SCC4 migration is enhanced by the synergistic effects of the mitogenic peptides on NOFs.

The effect of combining treatment of ET-1, Ang II and/or TGF-β on the phenotype of NOFs was investigated using a wound healing model. The combined treatment of NOFs with ET-1 and Ang II and Ang II and TGF-β resulted in a significant increase in their migration in comparison to their treatment with the peptides individually. The treatment of NOFs with ET-1 and TGF-β and all three peptides combined resulted in a small increase in their migration but not significantly more than that observed when the cells were treated with the peptides individually. This result was similar to that observed in the determination of the synergistic effects of the peptides on the stimulation of SCC4 migration via a paracrine mechanism. Some combined treatments resulted in an increase in NOF migration but not significantly more compared to treatment of the cells with the individual peptides. There was also an increase in α-SMA transcript levels for all combined in comparison to treatment of NOFs with each peptide individually. A similar pattern was observed when cells were subject to protein analysis.

These findings suggest that the effects of ET-1 and Ang II on NOFs can be enhanced when combined with other factors including peptides and cytokines. Porter and Turner (2009) identified that a similar synergism exists in lung tissue where ET-1 can act with TGF-β and Ang II to promote myofibroblast transdifferentiation.

These results again highlight the synergistic effect that the peptides have and their ability to further influence the behaviour of NOFs and promote their transdifferentiation into myofibroblasts. This result further complicates and highlights the importance of determining the factors present within the tumour microenvironment and the roles that individual and

combined components play in initiating and promoting tumour-stromal interactions. The results also suggest that the treatment of HNSCC may be more successful if numerous components were considered and targeted with combined drug therapies in order to counteract the synergistic and enhanced effects that the components including mitogenic peptides and cytokines promote as a combined unit.

7.6 Ang 1-7 can inhibit the autocrine and paracrine effects stimulated by mitogenic peptides

Angiotensin 1-7 (Ang 1-7), the major product formed by the catalytic degradation of Ang II, appears to have the ability to antagonise the effects triggered by Ang II stimulation (Herath *et al*, 2007). Ang 1-7 is produced from Ang II by angiotensin converting enzyme 2 (ACE2) at the Pro-7-Phe-8 bond. Ang 1-7 can also be hydrolysed from Ang I by NEP at the same bond (Chappell *et al*, 2000). The ability of NEP to hydrolyse Ang I tend to be masked by the activity of ACE2 (Herath *et al*, 2009). Ang 1-7 can also be generated from Ang I via the generation of Ang 1-9, a pathway which involves both ACE and ACE2 (Donoghue *et al*, 2000; Herath *et al*, 2007). *ACE2* was originally cloned from a human heart ventricular cDNA library (Tipnis *et al*, 2000). ACE2 knockout mice show a reduction in cardiac contractility which is an indicator of heart dysfunction (Crackower *et al*, 2002). This phenotype was rescued in the same study by the ablation of the *ACE* gene, highlighting a critical balance between the two enzymes (Guy *et al*, 2008). Further studies that involved disruption of the *ACE2* gene resulted in mice that gave viable, fertile progeny that did not express gross structural or anatomical abnormalities and showed moderately elevated systolic blood pressure (Gurley *et al*, 2006). Treatment of these animals with Ang II resulted in an increase in blood pressure and decreased heart rate in comparison to control animals. Gurley *et al* (2006) concluded that the absence of *ACE2* resulted in mice being more susceptible to hypertension triggered by Ang II. qPCR analysis determined that ACE2 mRNA was expressed in primary human NOKs and cell lines derived from HNSCC primary carcinomas, dysplasias and a local metastasis. ACE2 was not detectable within primary human NOFs. As mentioned before ACE was over expressed in NOFs in comparison to NOKs. Transcripts of the enzyme were present in all cell lines deriving from primary HNSCCs, oral dysplasias and a local metastasis. The distinct patterns of ACE and ACE2 observed in this study and work previously conducted highlights the possible importance in the balance of the two enzymes and the role that a change in their expression profiles may play in the progression of HNSCC. The balance of the two enzymes is critical in maintaining a correct and functioning local tissue RAS, in particular the production and hydrolysis of Ang II within physiological conditions (Lambert *et al*, 2010).

Ang 1-7 exerts its effects through the Mas receptor (MasR) (Santos *et al*, 2008; Santos *et al*, 2003). MasR mRNA was detectable in human primary NOKs and NOFs and cell lines derived from oral dysplasias and a local metastasis as identified by qPCR. The receptor was greatly over expressed in cell lines isolated from HNSCC primary carcinomas. This result is in keeping with the original identification of the MasR as an oncogene (Santos *et al*, 2003: Santos *et al*, 2008). The MasR is a seven transmembrane GPCR and was identified as having tumourigenic properties which originated from the rearrangement of its 5' flanking region (Young *et al*, 1986; Rabin *et al*, 1987). Studies conducted by Jackson *et al* (1988) identified that the tumourigenic properties exhibited by the MasR were negligible and their work using transfection procedures concluded that the *Mas* gene could encode for an Ang II receptor. This theory was not upheld and Ambroz *et al* (1991) identified that Ang II only induced intracellular Ca^{2+} responses in cells transfected with *Mas* if they already expressed endogenous levels of the AT_1R. Santos *et al* (2003) identified that the MasR binds Ang 1-7. They did this using radioligand binding, cell-specific binding and functional studies as well as creating an *in vivo* mouse model that was deficient in *Mas.* Although the MasR is known to bind Ang 1-7, some researchers have suggested that an indirect link and interaction between Ang 1-7 and the Ang II peptide and AT_1R cannot be excluded (Santos *et al*, 2003). Its overexpression in HNSCC cell lines suggests that it may play a ligand independent role in the progression of HNSCC progression.

Work conducted on vascular smooth muscle cells first identified that Ang 1-7 could oppose the mitogenic effects triggered by Ang II. Studies conducted *in vivo* and *in vitro* showed that Ang -1-7 could inhibit vascular smooth muscle cell proliferation, could reduce neointimal formation in the carotid artery after vascular injury and in the abdominal aorta after stent implantation (Freeman *et al*, 1996; Strawn *et al*, 1999; Langeveld *et al*, 2005). The ability of Ang 1-7 to inhibit the effects triggered by Ang II in cancer was also determined. The use of inhibitors to ACE which result in an increase in Ang 1-7 reduce the risk of cancer, particularly within the breast and lung (Lever *et al*, 1998). Ang 1-7 can reduce lung cancer cell migration (Gallagher and Tallant, 2004) and lung tumour growth which coincides with a reduction in COX-2 expression (Menon *et al*, 2007). In lung and breast cancer the peptide has also been shown to be able to inhibit microvessel density as a result of decreased levels of VEGF (Soto-Pantoja *et al*, 2009; Soto-Pantoja *et al*, 2008). Cook *et al* (2011) identified that Ang 1-7 can inhibit the growth of myofibroblasts and can reduce fibrosis within the breast tumour microenvironment. The peptide achieves this by reducing collagen 1 deposition and perivascular fibrosis. In this study Ang 1-7 reduced TGF-β signalling, fibronectin production and ERK 1/2 activity (Cook *et al*, 2011). Ang 1-7 also reduced MAPK signalling and Cook *et al* (2011) suggest that this is as a

result of the heptapeptide inducing an increase in dual specificity protein phosphatase 1 (DUSP1), a MAPK phosphatase.

Given the documented ability Ang 1-7 to oppose the pathophysiological effects of Ang II in other physiological settings, it was deemed appropriate to investigate the effect of Ang 1-7 on the migration and invasion of HNSCCs triggered by both aurocrine and paracrine stimulation by Ang II. The presence of Ang 1-7 inhibited Ang II stimulated migration and invasion of SCC4 cells suggesting that the peptide can antagonise the effects of Ang II in HNSCC. Ang 1-7 only inhibited HNSCC migration in the presence of Ang II. This is in contrast to the effect observed in breast and lung cancer, in which Ang 1-7 had a tumour suppressive effect in the absence of stimuli; these effects were observed within *in vivo* models (which may be constitutively stimulated by serum components and other factors) which may explain the difference between them and the result within this study. Within an *in vivo* scenario there may be increased levels of endogenous Ang II on which Ang 1-7 may be able to act and inhibit the effects of the mitogenic peptide. The pre-treatment of SCC4 cells with an inhibitor to the MasR resulted in the abrogation of Ang 1-7 inhibition of Ang II stimulation migration, suggesting that the peptide is exerting its effect through this receptor.

This is an intriguing paradox as Ang 1-7 appears to mediate its tumour suppressor activities through a receptor over-expressed on cancer cells. It is possible that the MasR has other, ligand independent, roles or unidentified ligands that can bind to and activate it. The MasR was initially identified as an orphan GPCR. It has been suggested that the control of non-orphan GPCRs may be controlled by positive and negative regulation by orphan GPCRs (Levoye *et al*, 2006). The Mas-related gene (Mrg) family is comprised of orphan and non-orphan GPCRs (Levoye *et al*, 2006). Mrg-D subtype is a non-orphan GPCR that is activated by β-alanine. Mrg-E is an orphan GPCR. Both GPCRs have been found as a heterodimer within human embryonic kidney (HEK) 293 cells. The presence of the heterodimer results in increased potency of β-alanine-stimulation of the Mrg-D subtype which leads to the phosphorylation downstream of ERK 1 and 2 and the maintenance of elevated levels of intracellular Ca^{2+} (Levoye *et al*, 2006). Levoye *et al* (2006) have also suggested that orphan GPCRs may also be able to recruit additional intracellular regulators to the heterodimer complex. Orphan GPCRs have also been identified as being constitutively activated and are therefore described as being ligand independent (Rosenkilde *et al*, 2006; Vischer *et al*, 2006). Constitutively activated orphan GPCRs can cause patho-physiological consequences due to their stimulation of constitutively downstream signalling (Schoneberg *et al*, 2004). In general the activity of orphan GPCRs is controlled not by ligand stimulation but maybe by regulating the actual protein levels of the receptor (Levoye *et al*, 2006). This control of protein levels may be achieved by the induction

of target-cell genome-encoded receptors (Levoye *et al*, 2006). Levoye *et al* (2006) also suggest that aside from controlling the level of orphan GPCR, the relative expression of the orphan GPCR compared with the paired non-orphan GPCR may also be another important mode of regulation. The orphan GPCR could also be controlled by post-translational modifications that are associated with GPCR desensitization (Levoye *et al*, 2006).

Although the MasR is no longer classified as an orphan GPCR, the characteristics that led researchers to first identify it as an oncogene and it's over expression in HNSCC cell lines and NOFs in comparison to NOKs requires further investigation to fully explain its role. The mechanisms via which the MasR functions are not fully elucidated and further work is required in order to discover if it can still function as an orphan-like receptor alongside its role as a receptor for Ang 1-7.

In order to investigate the ability of the peptide to antagonise the paracrine effects triggered by the treatment of NOFs with Ang II, the cells were pre-treated with the peptide before treatment with Ang II. Treatment of NOFs with Ang 1-7 only resulted in a slight increase in migration of the SCC4 cells. When NOFs were pre-treated with Ang 1-7 before the addition of Ang II there was a reduction in SCC4 migration, similar to that observed when the HNSCC cells were treated directly. A similar inhibition was observed for Ang II stimulated SCC4 cell invasion. These observations support the theory that Ang 1-7 can antagonise the paracrine stimulated mitogenic effects of Ang II.

The pre-treatment of NOFs with an antagonist to the MasR before the combined treatment with Ang 1-7 and Ang II resulted in the loss of the inhibition of Ang II stimulated migration by Ang 1-7. This finding suggests that Ang 1-7 is exerting its inhibitory effects through the MasR which is a similar result to that observed for the autocrine mechanism that was described previously within this thesis. The method by which Ang 1-7 inhibits the effect of Ang II is unclear. A number of studies have identified signalling pathways which Ang 1-7 can interfere with. In studies on the kidney, treatment with Ang 1-7 resulted in reduced levels of p38, ERK 1 and 2 and JNK phosphorylation and reduced TGF-β1 production caused by Ang II (Su *et al*, 2006). Ang 1-7 is also known to alter the levels of arachidonic acid metabolites including PGE_2 and the enzymes that regulate their production (Tallent *et al*, 1999; Tallant and Clark, 2003). Within this thesis mitogenic peptide paracrine stimulation of HNSCC migration coincides with an increase in COX-2 expression therefore highlighting a possible control point at which Ang 1-7 can act on and inhibit the activities triggered by mitogenic peptides. These all contribute to tumour progression if their control becomes unregulated. Diabetic patients experience enhanced EGFR phosphorylation at specific tyrosine residues and therefore an increase in ERK

1 and 2 and p38 MAPK downstream signalling (Akhtar *et al*, 2012). Rats treated with Ang 1-7 resulted in an inhibition of EGFR transactivation stimulated by both Ang II and glucose. The attenuated effects were inhibited upon treatment with a selective MasR antagonist. This evidence links Ang 1-7 with components that are important in the paracrine mechanism and stimulation of HNSCC by Ang II. This highlights the possibility of numerous control points within HNSCC and the tumour microenvironment.

Reports have identified that Ang 1-7 can reduce DNA, collagen and protein synthesis in cardiac fibroblasts after their stimulation with ET-1 or serum (McCollum *et al*, 2012). The effects triggered by Ang 1-7 can be inhibited when a receptor antagonist to the MasR is used (McCollum *et al*, 2012). The treatment of cardiac fibroblasts with Ang 1-7 after ET-1 or Ang II stimulation resulted in a decrease in phospho –ERK 1 and 2 stimulated by the mitogenic peptides (McCollum *et al*, 2012). Ang 1-7 also caused a decrease in COX-2 and prostaglandin synthase transcript levels which were induced and stimulated after treatment of the cardiac fibroblasts with ET-1 (McCollum *et al*, 2012). The effect of Ang 1-7 on the paracrine stimulation of HNSCC migration by other mitogenic peptides was investigated. NOFs were pre-treated with Ang 1-7 for 30 min before the addition of Ang II, ET-1, BK or EGF. NOFs treated with both Ang 1-7 and ET-1 or BK resulted in a decrease in SCC4 migration in comparison to that observed when NOFs were treated with the mitogenic peptides alone. This reduction in migration was significant but was not to the same extent as that observed when NOFs were treated with both Ang 1-7 and Ang II. The combined treatment of NOFs with Ang 1-7 and EGF did not result in a change in SCC4 migration. This result suggests that Ang 1-7 is not exerting its effects by inhibiting the ability of EGF to bind to the EGFR. EGF is known to bind directly to the EGFR, causing its activation (Carpenter and Cohen, 1990). ET-1, BK and Ang II are known to exert their effects by binding to GPCRs. This binding event can trigger the activation of members of the ADAMs family of cell surface proteinases including ADAM17, resulting in the release of soluble factors from the cell surface which has been observed in this study. The release of these stimulatory factors allows them to act as ligands for the EGFR. The reduction in SCC4 cell migration observed when Ang 1-7 is combined with ET-1 or BK suggests that the peptide could be interfering in the activation of members of the ADAMs family that would normally be triggered by the binding of ET-1 and BK to their specific GPCRs. Ang 1-7 could inhibit the activation of GPCRs therefore resulting in a decrease in the soluble factors being released from the surface of the NOFs. This event would result in a reduction in the number of potential ligands for the EGFR resulting in reduced activation of the receptor and a decrease in the stimulation of SCC4 cellular migration. The effect of combining Ang 1-7 with Ang II showed the greatest inhibition of SCC4 migration. It is known that Ang II can trigger the activation of

members of the ADAMs family in the same way that ET-1 and BK can but the greater effect on inhibition could also suggest that Ang 1-7 is targeting the effects triggered by Ang II at another specific location, perhaps interfering directly with the activation of AT_1R. Ang 1-7 could be exerting its inhibitory effects through the specific GPCRs to ET-1, BK and Ang II but the peptide may have a great affinity for AT_1R therefore explaining the increased inhibition observed when cells are treated with both Ang 1-7 and Ang II. This work will be the subject of further investigation.

Ang 1-7 is currently in Phase I clinical trials. Petty *et al* (2009) have conducted a study in order to determine its toxicity and pharmacokinetics in cancer patients. Their study concluded that treatment with Ang 1-7 is well-tolerated and a number of patients showed some clinical benefit. Their work concludes that the RAS is a potential and beneficial target in the development of drug therapies for the treatment of cancer. Work conducted in this thesis highlights that the system could be targeted in the treatment of head and neck cancer. Petty *et al* (2009) also stated the potential of the RAS as an important target for cancer chemoprevention. A number of large, clinical studies have concluded that ACE inhibitors can increase levels of the Ang 1-7 and have apparent chemopreventive activity (Luque *et al*, 1996; Pahor *et al*, 1996; Jick *et al*, 1997; Lever *et al*, 1998; van der Knaap *et al*, 2008). This study identifies and supports the potential benefits associated with the treatment of cancers with Ang 1-7. The peptide not only has the ability to target epithelial cancer cells but also components within the surrounding tumour microenvironment further expanding the opportunities of the peptide as a possible therapeutic treatment in cancer.

Chapter 8: Conclusion and Future Work

8.1 Conclusion

The results obtained in this thesis provide compelling evidence for a role for mitogenic peptides in promoting head and neck cancer progression.

Endothelin-1 (ET-1) and angiotensin II (Ang II) are both examples of mitogenic peptides and are both regulated via a complex system of enzymes and specific receptors. The expression levels of ET-1 are controlled by the ET-axis which consists of the enzymes endothelin converting enzyme-1 (ECE-1) and neprilysin (NEP) and its two specific cell surface receptors ET_AR and ET_BR. Ang II is similar to ET-1 in that two enzymes also control its expression, angiotensin converting enzyme (ACE) and angiotensin converting enzyme 2 (ACE2), and the peptide can bind to two specific G protein-coupled receptors (GPCRs); AT_1R and AT_2R. These controlling components are known collectively as the renin angiotensin system (RAS). The RAS also contains an additional peptide angiotensin 1-7 (Ang 1-7), derived from Ang II, which is thought to have antagonise the effects triggered by the binding of Ang II to AT_1R.

The data collected within this study suggests that mitogenic peptides can promote HNSCC migration and invasion via distinct mechanisms. ET-1 and Ang II both work via a paracrine mechanism that involves the binding of the peptides to their specific GPCRs on neighbouring cells. The activation of their GPCRs on the surface of normal oral fibroblasts (NOFs) allows the activation of ADAM17 which results in the release of soluble factors including tumour growth factor-α (TGF-α), amphiregulin and heparin bound-epidermal growth factor (HB-EGF). These factors are released from the NOFs and can act as ligands for the EFR receptor (EGFR) located on the cancer cells. The activation of the EGFR ultimately results in the triggering of intracellular signalling pathways stimulating signalling pathways resulting in the upregulation of cyclooxygenase-2 (COX-2) and promoting an increase in HNSCC cellular migration and invasion. Ang II can also stimulate head and neck squamous cell carcinoma (HNSCC) via an autocrine mechanism similar to that observed for bradykinin (BK) in the promotion of head and neck cancer (Thomas *et al*, 2006). The ability of ET-1 and Ang II to stimulate head and neck cancer progression proves the hypothesis first set out at the beginning of this thesis. The peptides promote HNSCC progression via the stimulation of epithelial cancer migration and invasion; two characteristic hallmarks associated with the progression of cancer.

In addition, experiments conducted within this thesis have implicated microRNAs (miRNAs) in modulating peptide-stimulated stromal-epithelial interactions and the findings have highlighted the important and complex role that miRNAs can play in the regulation of factors including ADAM17 that are known directly and indirectly to promote cancer progression. It is

necessary to fully determine the characteristics that are changed upon the activation of fibroblasts and how these changes alter the cells ability to promote cancer progression.

The final aim of this thesis was to investigate the role of the tumour microenvironment in head and neck cancer progression. The results of this study have highlighted the importance of the tumour microenvironment which surrounds cancer cells in modulating the pro-migratory and pro-invasive effects of mitogenic peptides. This project has begun to elucidate the molecular mechanisms underlying tumour-stromal interactions between fibroblasts cells, the most numerous cell type found within the reactive stroma, and epithelial HNSCC cells. This work has, to date, mainly utilised fibroblasts isolated from non-cancerous specimens. *In vivo*, fibroblasts in proximity to cancer cells acquire an activated, myofibroblast phenotype. This project has also begun to address the ability of the mitogenic peptides to influence the phenotype of NOFs. The mechanism by which ET-1 stimulates phenotypic changes in NOFs remains unclear. In this study, unlike TGF-β, a well characterized stimulant of myofibroblast transdifferentiation, and Ang II, ET-1 did not significantly alter the expression of α-smooth muscle actin (α-SMA) and matrix metalloproteinase (MMP)-2, two 'markers' of myofibroblasts.

This is the first report of Ang II-mediated fibroblast remodelling influencing cancer cell behaviour. The ability of ET_AR and ET_BR antagonists and an AT_1R antagonist to block the fibroblast phenotypic changes induced by ET-1 and Ang II, at least *in vitro*, raises the possibility of utilising such antagonists in the clinic to inhibit metastasis and to reduce fibrosis to improve penetration of existing chemotherapeutic agents.

This study has identified that Ang- 1-7 can inhibit the autocrine and paracrine stimulation of HNSCC migration and invasion triggered by Ang II, ET-1 and BK. This novel finding and studies which have indicated that in clinical trials Ang 1-7 is well-tolerated and shows some clinical benefit highlight the potential use of the peptide as a therapeutic agent in the treatment of head and neck cancer.

The survival rate of HNSCC patients has increased only slightly over the past three decades. The work conducted in this thesis has identified novel mechanisms that contribute to HNSCC progression and has therefore highlighted possible new therapeutic targets and clinical applications that could be used in the diagnosis and treatment of the disease.

8.2 Future work

The work carried out in this thesis and the results collected have raised a number of questions. The carrying out of future experiments which would focus on answering these questions would allow further conviction and validation of the results already collected and would allow further progress to be made in determining the role mitogenic peptides play in HNSCC progression.

Analysis of tissue from patients

Staining of tissue collected from HNSCC tumours and normal tissue would allow further evaluation and confirmation of the components involved in HNSCC cancer progression. The enzymes involved in the regulation of ET-1 and Ang II could be stained for in order to establish their localization and any changes that occur in their expression levels during the different stages of cancer development. Staining for the expression of the specific GPCRs for ET-1 and Ang II needs to be conducted to further analyse the methods by which the mitogenic peptides are contributing to the promotion of HNSCC cancer progression. By performing staining on a number of different patient samples a true representation of altered receptor expression can be concluded. RNA could also be extracted from tissue from the same patient cohort. This would allow qPCR analysis to be conducted on a number of patient samples thereby providing information on receptor and enzyme expression *ex vivo*.

Tumour microenvironment

From the work conducted in this thesis the importance of the tumour microenvironment is apparent. The involvement of NOFs in cancer progression triggered by mitogenic peptides is imperative. Further work is needed to fully understand the extent to which the fibroblasts are involved in HNSCC progression.

Construction of a 3D organotypic model would allow better representation of a system more in keeping with that observed *in vivo*. The model could combine NOFs and HNSCC cells, recreating a microenvironment. The incorporation of other cell types would further improve the *in vitro* scenario making it more similar to that observed *in vivo*. The inclusion of immune cells or the involvement of endothelial cells would allow this improvement in comparisons between *in vitro* and *in vivo* scenarios. The results collected from the conduction of a 3D organotypic model would also allow the commencement of *in vivo* models in which coinjection of cancer cells and NOFs/cancer associated fibroblasts (CAFs) into immunocompromised mice would allow the assessment of the effects of GPCR antagonists and/or Ang 1-7.

Further analysis needs to be conducted in order to determine the importance of myofibroblasts within the tumour microenvironment and their ability to promote HNSCC

progression. The isolation and characterisation of CAFs from head and neck tumours would be invaluable. The use of fibroblasts isolated form HNSCCs which show an increase in α-SMA expression in future experiments instead of treating NOFs with TGF-β could help in the investigation of the importance of the tumour microenvironment and would allow the creation of an environment more similar to that observed *in vivo*. The isolated myofibroblasts could then be incorporated into experiments similar to those that have previously been conducted and any differences in HNSCC cell migration or invasion could be analysed. It is possible that the treatment of myofibroblasts with mitogenic peptides may promote the migration ability of HNSCC cells even further in comparison to that observed with NOFs.

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Appendices